



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 48/00, C12N 15/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 96/18419</b> <b>(43) International Publication Date:</b> 20 June 1996 (20.06.96)
<b>(21) International Application Number:</b> PCT/US95/16347 <b>(22) International Filing Date:</b> 14 December 1995 (14.12.95)  <b>(30) Priority Data:</b> 08/357,508           14 December 1994 (14.12.94)   US 08/476,257           7 June 1995 (07.06.95)       US 08/534,220           11 September 1995 (11.09.95)   US  <b>(71) Applicant (for all designated States except US):</b> UNIVERSITY OF WASHINGTON [US/US]; Office of Technology Transfer, Suite 200, 1107 N.E. 45th Street, Seattle, WA 98105 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> KAY, Mark, A. [US/US]; 8032 19th Avenue N.E., Seattle, WA 98115 (US). LIEBER, Andre [DE/US]; 1303 N.E. Campus Parkway #203, Seattle, WA 98105 (US).  <b>(74) Agents:</b> PARMELEE, Steven, W. et al.; Townsend and Townsend and Crew, Steuart Street Tower, 20th floor, One Market Plaza, San Francisco, CA 94105 (US).		<b>(81) Designated States:</b> AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> RECOMBINANT VECTORS FOR PERMANENT RECONSTITUTION OF LIVER AND TREATMENT OF HEPATITIS C		
<b>(57) Abstract</b> <p>A combination of retroviral and adenoviral vectors are used for high efficiency gene transfer into hepatocytes, resulting in long term gene expression. Hepatocytes are transduced <i>in vivo</i> with a recombinant adenovirus vector that expresses a molecule capable of inducing hepatocyte regeneration, such as urokinase plasminogen activator (uPA) or tissue plasminogen activator (tPA), resulting in a high rate of liver regeneration. During the regenerative phase, <i>ex vivo</i> or <i>in vivo</i> retroviral-mediated gene transfer into hepatocytes results in greater transduction efficiencies. The compositions and methods thus provide new means for gene therapy, and transgenic non-human animals useful in developing new therapeutic and preventative agents. The vectors can be used for high efficiency transduction of ribozymes specific for hepatitis C virus RNA.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5

## RECOMBINANT VECTORS FOR PERMANENT RECONSTITUTION OF LIVER AND TREATMENT OF HEPATITIS C

10

### Related Applications

15 This is a continuation-in-part of USSN 08/534,220, filed September 11, 1995, which is a continuation-in-part of USSN 08/476,257, filed June 7, 1995, which is a continuation-in-part of USSN 08/357,508, filed December 14, 1994, each of which is incorporated herein by reference.

### Government Support

20 Certain embodiments of the invention described herein were made in the course of work supported by the National Institutes of Health pursuant to grant no. DK47754. Therefore, the U.S. Government has certain rights in this invention.

25

### Background Of The Invention

30 The major etiological agent of posttransfusional and community acquired non-A non-B hepatitis has been identified as hepatitis C virus (HCV). Choo et al., Science 244: 359-362 (1989). At present, intravenous drug abuse is the most important risk factor for transmission of HCV. However, different epidemiological studies have revealed that for up to 35 20 to 40% of patients chronically infected with HCV, no known risk factors for HCV can be demonstrated. Alter et al., N. Engl. J. Med. 327:1899-1905 (1992).

Although the disease associated with HCV may be benign, persistent infection may lead to liver cirrhosis and hepatocellular carcinoma (Saito et al., Proc. Natl. Acad. Sci. USA 87:6547-6549), although the mechanism of cellular transformation is unknown. HCV disease can be manifested as acute viral hepatitis which is usually clinically mild, but in other cases the disease may develop into a severe or fulminant hepatitis. Chronic HCV hepatitis is believed to occur more frequently than with hepatitis B virus, especially following posttransfusional acute hepatitis C disease, i.e., in about 54% of cases. Hollinger, in Fields Virology, 2d ed., Chpt. 78, eds. B. Fields and D. Knipe, Raven Press, NY (1990).

On the basis of sequence homology, the single-stranded positive-sense RNA enveloped HCV virus has been provisionally classified as a separate genus of the family Flaviviridae. Miller and Purcell, Proc. Natl. Acad. Sci. USA 87:2057-2061 (1990). The HCV genome is about 10 kb in length and it encodes a single polyprotein of about 3,000 amino acids that includes structural and nonstructural proteins that are processed by cellular and virus-encoded proteinases. The processed gene products include a putative core (C), three putative envelopes (E1, E2 type A, and E2 type B), and six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Representative sequences of HCV strains are described in U.S. Patent 5,350,671 to Houghton et al., incorporated herein by reference.

Comparative sequence analysis of complete HCV genomes (Okamoto et al., Virology 188:331-341 (1992)) and PCR fragments from various genomic regions has shown that HCV may be grouped into distinct but related genotypes. At present, six major genotypes (1-6) with numerous subtypes (e.g., 1a, 1b; 2a, 2b, 3a, 5a) have been identified. Three additional types have been recently identified but are apparently limited in geographic distribution. Some genotypes have been associated with severity of disease (Pozzato et al., J. Med. Virol. 43:291-296 (1994)) and responsiveness to interferon therapy (Yoshioka et al., Hepatology 16:293-299 (1992)).

To date, treatment of HCV infection has primarily been with alpha-interferon. In some instances liver transplantation has been performed for end-stage hepatic deficiency, but invariably the transplanted liver also becomes infected with HCV and ultimately fails.

Gene therapy involves the introduction of genetic material into the cells of an organism to treat or prevent a disease. The material transferred can be from a few nucleotides to a few genes in size. Gene therapy is potentially useful in the treatment and prevention of acquired and hereditary diseases, such as infectious diseases, cancer, etc. A variety of cell types have been targeted in somatic cell gene therapy systems, including hematopoietic cells, skin fibroblasts and keratinocytes, hepatocytes, endothelial cells, skeletal and smooth muscle cells, and lymphocytes, each with varying success.

Methods for gene therapy involving the liver have often relied on gene transfer ex vivo, i.e., inserting genes into hepatocytes which have been removed from a patient, and are then reimplanted into the liver. Because primary cultures of hepatocytes cannot be expanded, gene transfer must occur at high efficiency to obtain suitable numbers of cells for reimplantation. Although long term expression of foreign genes in transduced hepatocytes has been accomplished with retroviral vectors, the efficiency of retroviral transduction is relatively low, as retrovirus infects only dividing cells (Miller et al., Mol. Cell. Biol. 10:4239-4242 (1990)), and the protein may not be expressed in therapeutically or prophylactically effective amounts. In one ex vivo method approximately 20% of a patient's liver is surgically removed, the cells are then transduced with the retroviral vector, and then implanted back into the patient. This approach suffers from obvious disadvantages of surgical procedures and a low efficiency of transduction and expression of the gene product of interest.

Similarly, an in vivo approach to transducing hepatocytes with retroviral vectors involves first performing a partial hepatectomy, which is followed by portal vein

infusion of the vector. The removal of the majority of the liver is needed to stimulate liver regeneration so that the infused retrovirus will integrate into a sufficient number of the hepatocyte genomes. As with the ex vivo approach, this method suffers from requiring a major surgical procedure and under the best of conditions only about 1% of the liver mass contains the genetically modified vectors.

As an alternative to retroviral-mediated hepatic gene therapy, the adenovirus presents a transfer vector that can infect nonreplicating cells at high efficiency. Adenoviral DNA remains extra-chromosomal and thus is slowly lost from transduced hepatocytes over a period of several months. Li et al., Human Gene Ther. 4: 403-409 (1993); Kay et al., Proc. Natl. Acad. Sci.USA 91: 2353-2357 (1994).

Additionally, a substantial portion of the adenovirus is taken up by organs and tissues other than the liver, which may raise issues of safety. (Smith et al., 1993, and Kay et al., *ibid.*). And, as adenovirus stimulates the production of neutralizing antibodies in an infected host, patients who have been naturally infected with adenovirus may be resistant to gene therapy using this vector, or secondary transductions may be prevented by the presence of antibodies produced in response to a primary transduction (Smith *ibid.*, Kay, *ibid.*).

The liver is a desirable target for somatic gene transfer because it is a large organ that is responsible for the synthesis, processing and secretion of many circulating proteins, including many of the plasma coagulation proteins. Because of the liver's involvement in many diseases of medical importance much effort has focused on replacing diseased livers by transplantation or, due to a severe shortage of donor livers, by implantation of healthy liver cells. Typically, however, implanted hepatocytes have made only small and temporary contributions to liver function.

Transgenic animal technology has been employed to create and analyze models of diseases affecting many organs, including the liver. A variety of transgenes have been reported to be associated with liver lesions, including urokinase-type plasminogen activator (uPA). In mice

containing a albumin-urokinase transgene the hepatocyte-targeted expression of the uPA gene created a functional liver deficit. The uPA gene caused the fatal hemorrhaging of newborn mice, and survivors displayed hypofibrinogenemia and unclottable blood. It was thus concluded that any injury sufficient to initiate bleeding was rapidly fatal in affected mice. Heckel et al., Cell 62: 447-456 (1990). Surviving mice did show a gradual decrease in the level of plasma uPA activity, accompanied by a restoration of clotting function within one to two months. This was explained by a report that the uPA was cytotoxic for hepatocytes and that inactivation of transgene expression by DNA rearrangement in isolated hepatocytes in Alb-uPA mice was followed by repopulation of the entire liver by cells that no longer produce uPA. Sandgren et al., Cell 66: 245-256 (1991). The uPA transgene-expressing hepatocytes were at a selective disadvantage relative to hepatocytes (native or non-native) that were not expressing the transgene. Thus, production of uPA by the liver kills hepatocytes over time, and the gene encoding uPA has been used to impair native liver function and stimulate the repopulation of liver with non-native cells. See Brinster et al., PCT Publication WO 94/02601, and Rhim et al., Science 263: 1149-1152 (1994).

There remains a significant need in the art for methods of somatic gene therapy that use the liver for efficient expression of gene product in therapeutically useful quantities and duration. Desirably, these methods should (a) avoid the necessity for surgically removing a large portion of the liver, (b) enhance the yield and recovery of transduced hepatocytes without compromising viability; and (c) be independent of the particular disease being treated.

A related need exists in the art for compositions useful in treating hepatitis C infection and methods for their delivery to HCV-infected cells of the liver. Desirably, the compositions and methods should effectively reduce or eradicate HCV from infected cells, or should significantly impair the ability of the virus to replicate, thereby preventing further dissemination of the disease. The

compositions should be inherently specific for HCV and of negligible toxicity. Quite surprisingly, the present invention fulfills these and other related needs.

5

### Summary Of The Invention

10 In one aspect of the invention methods and compositions are provided for producing a gene product of interest in the liver of a mammal. In one embodiment native hepatocytes which have been removed from the mammal are transduced with a vector which encodes the gene product of interest, and native hepatocytes of the mammal are transduced  
15 in situ with an adenoviral vector which encodes a molecule that stimulates liver regeneration. Preferably the molecule is uPA, which can be modified to inhibit its secretion from the hepatocyte when expressed by the viral vector. In another embodiment the vector encodes tPA, which stimulates hepatocyte  
20 regeneration de novo, apparently without hepatocyte killing. The transduced hepatocytes which have been removed from the mammal are returned to the mammal, and conditions are provided which are conducive to expression of the gene product of interest. Typically the transduced hepatocytes are returned  
25 to the patient by infusion through the spleen or portal vasculature, and administration can be one or more times over a period of 1 to 5 days or more.

In an in vivo aspect of the methods of the invention, a retroviral, pseudotype or adenoviral associated  
30 vector is constructed which encodes the gene product of interest and is administered to the mammal in conjunction with a regeneration of the liver induced by an adenoviral vector that encodes tPA, which stimulates hepatocyte regeneration without hepatotoxicity, or a secretion-impaired hepatotoxin  
35 such as the modified uPA described herein. This method avoids the need for surgery to remove hepatocytes from the mammal prior to administering the adenovirus vector encoding the tPA or a hepatotoxin. An additional gene can be included in the



vector which encodes the gene product of interest, which additional gene encodes a molecule capable of binding to and inactivating the tPA or hepatotoxin encoded by the adenovirus vector.

5 A wide variety of gene products of interest may be administered to a mammal according to the invention, often to treat or prevent a disease associated with the gene product. For example, the gene product of interest can be an enzyme, hormone, cytokines, antigens, antibodies, clotting factors, 10 anti-sense RNA, regulatory proteins, ribozymes, fusion proteins and the like. The methods can thus be used to supply a therapeutic protein such as Factor VIII, Factor IX, erythropoietin, alpha-1-antitrypsin, insulin, interferons, colony stimulating factor, interleukins, G-CSF, GM-CSF, M-CSF, 15 adenosine deaminase, etc.

In another aspect the invention provides non-human transgenic animals which contain a gene encoding a modified, non-secreted hepatotoxin such as the secretion-impaired uPA.

Methods and compositions are also provided for 20 producing ribozymes targeted to HCV gene products of interest in an HCV-infected or susceptible mammal, thereby inhibiting or preventing HCV infection and spread. In one aspect the methods comprise transducing cells, especially hepatocytes, with a recombinant adenovirus which encodes a ribozyme 25 specific for hepatitis C virus RNA. When the sequence encoding the ribozyme is expressed, HCV RNA in the infected cell is inhibited or infection is prevented. Preferably the ribozyme is a hammerhead ribozyme, and specifically cleaves hepatitis C RNA in a HCV 5'non-coding sequence, core sequence, 30 or NS-5 sequence. Administration may be performed with hepatocytes from an HCV-infected individual, i.e., ex vivo administration, or administered to the individual. When administered to an individual, typically administration will be by infusion, such as via the portal vein or bile duct. 35 Typically the recombinant adenovirus is administered to the hepatocytes in an amount and for a time sufficient to eradicate hepatitis C virus from the hepatocytes, preferably from about 10 to 100 adenovirus particles per hepatocyte. For

increased activity against HCV, the vector(s) may encode two or more ribozymes specific for different regions or strands of HCV RNA.

In another aspect the invention provides recombinant adenovirus encoding at least one ribozyme specific for HCV RNA, and sometimes will encode two different ribozymes specific for HCV RNA. Preferably the recombinant adenovirus encodes a hammerhead ribozyme specific for HCV. In yet other embodiments a ribozyme specific for HCV RNA targets an HCV sequence that corresponds to a cDNA sequence comprising

CGGGAGGTCTCGTA [Seq. ID:1] (nucleotide 317 to 332),  
GCACCATGAGCACGAA [Seq. ID:2] (nucleotide 337 to 352),  
CTAAACCTCAAAGAAA [Seq. ID:3] (nucleotide 355 to 370),  
CCAACCGTCGCCACAG [Seq. ID:4] (nucleotide 385 to 401),  
CCCACAGGACGTCAAG [Seq. ID:5] (nucleotide 385 to 410), or  
GGTAAGGTCATCGATA [Seq. ID:6] (nucleotide 699 to 714).

#### Brief Description of the Figures

Fig. 1A and Fig. 1B show the effects of Ad.RSV-uPA mediated gene transfer. Fig. 1A shows the serum concentrations of uPA, PT and SGPT at different times after Ad.RSV-uPA administration (n=5 per point, vertical lines represent the standard deviation). Fig. 1B shows 3H-thymidine uptake into liver DNA after Ad.RSV-uPA, Ad.RSV-hAAT or partial hepatectomy (n=3 animals per point, and vertical lines represent standard deviations).

Fig. 2A-G collectively demonstrate the effect of retrovirus-mediated gene transfer. Mice were subjected to a 2/3 partial hepatectomy or portal vein infusion of  $5 \times 10^9$  pfu of recombinant adenovirus on day 0. On varying day(s), the mice were infused (portal vein) with 1 ml of (A-F) LNAIbhAAT or (G) LTR-cFIX retrovirus. Blood samples were periodically analyzed for serum hAAT or cFIX by ELISA. Fig. 2A represents partial hepatectomy and LNAIbhAAT infusion 48 hours later; Figs. 2B-D show the effect of Ad.RSV-uPA and LNAIbhAAT

infusion on (B) day 3; (C) days 3, 5 and 7; and (D) days 5, 7 and 9. Figs. 2E and 2F show results of Ad.RSV-Bgal and LNAIbhAAT infusion on: (E) day 3; (F) days 3, 5 and 7. Fig. 2G shows results with mice infused with LTR-CFIX: 48 hours after partial hepatectomy, solid circles; on days 3, 5 and 7 after Ad.RSV-uPA administration, open circles. Each serum sample was analyzed in at least duplicate. Each line represents an individual animal.

Fig. 3A-C indicate the (A) uPA cDNA; (B) C-terminal modification; and (C) N-terminal modification.

Fig. 4A and B demonstrate the localization of uPA and modified uPAs in tissue culture cells. CHO cells were transduced with the indicated adenovirus vectors. Supernatants and cell lysates were analyzed for: (Fig. 4A) Antigen levels by ELISA (ng antigen/ $1 \times 10^6$  cells for supernatants or cell lysates). (4B) Enzymatic activity expressed as units/ $1 \times 10^6$  cells for supernatants or lysates. Each experiment was performed in duplicate.

Fig. 5 illustrates measurements of serum SGPT, prothrombin times (PT), uPA antigen in liver and serum from mice given  $5 \times 10^9$  pfu of Ad.PGKANAC-uPA adenovirus by portal vein infusion. The vertical bars represent the standard deviation.

Fig. 6 shows hepatic  $^3\text{H}$ -thymidine uptake. Mice were infused with  $5 \times 10^9$  pfu of Ad.PGKANACuPA adenovirus and at various times the animals were infused with  $^3\text{H}$ -thymidine at 24h, 12h and 45 minutes prior to sacrifice. The specific activity was expressed as cpm/ $\mu\text{g}$  DNA. The vertical lines represent the standard deviation. At least three animals were analyzed per time point. For comparisons animals receiving partial hepatectomy or Ad.RSV-uPA were included.

Fig. 7A and 7B demonstrate results of retrovirus-mediated gene transfer, where mice were infused with  $5 \times 10^9$  pfu of (Fig. 7A) Ad.RSV-uPA or (Fig. 7B) Ad.PGKANAC-uPA and 1 ml of LNAIbhAAT ( $2 \times 10^6$  cfu) retrovirus on days 3 and 5 after adenovirus infusion. Serum was periodically quantitated for hAAT in at least duplicate. Each line represents an individual animal.

Fig. 8 shows adenovirus ribozyme expression cassettes. The ribozymes are cloned into the pXCJL.1 plasmid to produce the recombinant adenovirus vectors. The promoters used to drive expression of the ribozymes (Rz) are also shown.

Fig. 9 shows cleavage sites for HCV ribozymes Rz1-Rz6 on the HCV RNA plus and minus strands.

Fig. 10 shows the effect of adenovirus-mediated gene transfer of HCV ribozymes into HCV-RNA cell lines. The values represent the mean and standard deviations for five different experiments. Ribozymes 2 and 3 are directed against the negative HCV RNA strand and have no specific effect on plus strand HCV RNA concentrations.

Fig. 11A shows the concentration of human growth hormone serum concentrations in hGH-transgenic mice after ribozyme therapy. Serum samples were analyzed for growth hormone by ELISA analysis at varying times after recombinant adenovirus administration. Fig. 11B shows hepatic mRNA quantitation in transgenic mice after ribozyme therapy. Ad/RSVhAAT is a control vector; Ad.polIII.Rz is adenovirus containing the hGH ribozyme driven by the adenovirus vaI promoter; Ad.T7Rz+Ad.T7 pol is a 1:1 mixture of the adenovirus expressing nuclear T7 polymerase from the PGK promoter and an adenovirus containing the T7 promoter used to drive transcription of the hGH ribozyme.

Fig. 12 shows the effect of ribozymes on HCV-RNA expression in HCV-infected human hepatocytes, where HCV RNA is shown in pg/10<sup>5</sup> cells.

#### Description Of The Specific Embodiments

In one aspect the present invention provides methods and compositions for somatic cell gene therapy which is targeted to the liver of a mammal of interest. The gene which is transduced into the hepatocytes of the animal can be expressed in a stable and prolonged manner, thereby providing

the animal with an ample supply of gene product to treat or prevent a selected disease.

In one embodiment the invention comprises an ex vivo method of hepatic gene therapy. According to this embodiment a collection of hepatocytes are removed from the individual, typically by surgical or biopsy means. Although a small amount of hepatocytes are necessary, e.g., about  $1 \times 10^7$ , sometimes about  $1 \times 10^8$ , sometimes about 1 to  $5 \times 10^9$  cells, or up to 20% or more (but desirably less) of the patient's liver can be removed and serve as a source of cellular material for transduction. The hepatocytes are obtained by collagenase liver perfusion and optionally cultured or and prepared for transduction by a vector which contains a gene that expresses the gene product of interest.

The vector can be prepared in any of a wide variety of ways. Typically, the vector will be a retroviral vector and will contain at least one viral long terminal repeat and a promoter sequence upstream of and operably linked to a nucleotide sequence encoding the gene product of interest, followed by at least one viral long terminal repeat and polyadenylation signal downstream of the sequence encoding the gene product of interest. Representative retroviral vectors suitable for use in the present invention are described, for example, in U.S. Patent Nos. 4,861,719, 5,124,263 and 5,219,740, Kay et al., Hum. Gene Ther. 3: 641-647 (1992) and Kay et al., Science 262: 117-119 (1993), each of which is incorporated herein by reference. Other vectors may also be employed, particularly for the ex vivo methods described herein, such as DNA vectors, pseudotype retroviral vectors, adeno-associated virus, gibbon ape leukemia vector, VSV-G, VL30 vectors, liposome mediated vectors, and the like.

In one embodiment the vector encoding the gene product of interest is used to transduce hepatocytes which have been isolated from the patient. In some cases, e.g., extreme hepatocellular disease, it may be desirable to use hepatocytes which have been isolated from an individual which is a suitable hepatocyte donor, i.e., one who is substantially the same or closely related in histocompatibility type. The

transduced hepatocytes may be cultured for up to 5 to 10 days or longer before being returned to the patient, but typically the cells will be returned to the individual by infusion, typically via the portal or splenic vein, in single or multiple administrations, within 1-5 days after removal.

Prior to reinfusing the transduced hepatocytes to the patient, the patient is infected with an adenoviral vector which encodes a hepatotoxic protein, e.g., one capable of inhibiting or slowly killing hepatocytes, or alternatively, a hepatocytic growth factor such as hepatocyte growth factor, or other factor which is capable of inducing hepatocyte proliferation, e.g., epidermal growth factor or the like. Typically the protein is one such as urokinase-type plasminogen activator (uPA), or the protein can be tissue-type plasminogen activator (tPA), which can stimulate hepatocyte regeneration de novo, without causing liver damage. The hepatotoxin should be specific for hepatocytes, or if not specific, should not be secreted by the infected hepatocytes into the bloodstream. A representative example is uPA which has been modified by N-terminal and/or C-terminal modifications as described herein so as to inhibit secretion by the infected host cell. In some cases other proteins may be used, where they are placed under the control of tissue-specific (liver) promoters and are not secreted, or the vectors are specifically targeted to hepatic tissue. These include proteins such as the cytotoxic domain of bacterial toxins such as *Pseudomonas* exotoxin A, diphtheria toxin, cholera toxin, shiga and shiga-like toxin, ribosome inactivating toxins derived from plants and fungi (e.g., ricin), hepatocyte growth factor, and others described in Genetically Engineered Toxins, ed. A. Frankel, Marcel Dekker, Inc. (1992), incorporated by reference herein.

Representative adenoviral vectors which can be used to encode the hepato-targeted gene are described in Stratford-Perricaudet et al., J. Clin. Invest. 90: 626-630 (1992), Graham and Prevec, in Methods in Molecular Biology: Gene Transfer and Expression Protocols, 7: 109-128 (1991) and Barr

et al., Gene Therapy, 2:151-155 (1995), each of which is incorporated herein by reference.

Because the adenovirus is capable of infecting dividing and non-dividing hepatocytes at high efficiency, a sufficient number of adenoviral vector particles should be administered to the liver to infect up to at least about 50% of the hepatocytes, usually about 80%, preferably about 95%, and more preferably 99% to 99.99% or more of the hepatocytes in the individual. In the ex vivo method the adenoviral-hepatotoxin vector is typically administered to the mammal as soon as possible after the hepatocytes have been removed, and prior to or simultaneous with the infusion of the hepatocytes which have been transduced to express the gene product of interest. The adenovirus vector can be administered by a variety of routes, but typically by intravascular infusion via portal vein, and from about 10 up to about 100 or more adenovirus particles per hepatocyte are administered.

The expression of the hepatotoxin encoded by the adenoviral vector can be constitutive or inducible, but typically is constitutive. As the adenovirus-infected hepatocytes which express the hepatocytes die or are inhibited, the hepatocytes which have been treated ex vivo to encode the gene product of interest are returned to the patient. As these cells have not been transduced with the hepatotoxin, they will proliferate and repopulate the liver. Thus, over a period of days to weeks the adenoviral-infected hepatocytes will die and the liver will be regenerated by hepatocytes which encode the gene product of interest.

Expression of the gene encoding the gene product of interest can be constitutive or inducible. If inducible, it will be under the control of a promoter which is different from a promoter which may be used to control expression of the hepatotoxin of the adenoviral vector. A supply of hepatocytes removed from the patient prior to adenoviral infection and which have been transduced by the vector, e.g., retrovirus, can be used to repeat the re-seeding of the liver as necessary. The entire process itself may be repeated as necessary, understanding that an immune response to the

adenovirus vector may be generated by repeated administration. The immune response may ameliorate the effect of adenoviral administration and thus necessitate administration of larger quantities of the vector or delivery in a manner by which the particles are shielded from the host's immune system, or the host's immune can be tolerized to the vector.

In another aspect the invention provides for in vivo methods of somatic cell therapy to the liver. According to this method a retroviral, pseudotype or adenoviral associated vector which requires cell division is constructed which encodes the gene product of interest. The vector is administered to the mammal in conjunction with the asynchronous regeneration of the liver which is or has been induced by an adenoviral vector that encodes the non-secreted hepatotoxin such as the modified uPA described herein. This method avoids the need for removing and transducing hepatocytes from the patient prior to administering the adenovirus vector encoding the hepatotoxin.

To increase the percentage of cells which express the gene product of interest in the in vivo method, an additional gene can be included in the vector which encodes the gene product of interest. This additional gene encodes a protein which is capable of binding to the hepatotoxin encoded by the adenovirus vector, and inactivates or inhibits the hepatotoxin's activity. For example, when the hepatotoxin is uPA or tPA the hepatotoxin inhibitor can be plasminogen activator inhibitor I or plasminogen activator inhibitor II.

According to this aspect of the in vivo method, the individual is treated with the adenoviral vector encoding the hepatotoxin as described for the ex vivo approach. The individual is simultaneously or subsequently treated with the retroviral vector encoding the disease-associated gene product of interest and the hepatotoxin inhibitor. Only the retroviral-transduced cells that produce the inhibitor and the disease-associated gene product of interest survive the effects of the hepatotoxin, and these cells selectively repopulate the liver. In yet another aspect the patient is first given a partial hepatectomy, then administered the



retrovirus encoding the gene product of interest simultaneously or prior to administration of the adenovirus encoding the hepatotoxin. In each of these methods the resulting liver is comprised of cells which express the disease-associated gene product of interest, thereby preventing or treating the disease in said patient.

As used herein, the terms "treatment" or "treating" cover any treatment of disease, and include: (1) preventing disease from occurring in a subject who does not have the disease or who has not yet been diagnosed as having it; (2) inhibiting or arresting the development of the disease; or (3) regressing or reversing the disease.

According to these methods a wide variety of disease-associated gene products of interest may be employed to treat or prevent the disease of interest. For example, and by way of illustration only, the genes can encode enzymes, hormones, cytokines, antigens, antibodies, clotting factors, anti-sense RNA, regulatory proteins, ribozymes, fusion proteins and the like. The methods can thus be used to supply a therapeutic protein such as Factor VIII, Factor IX, Factor VII, erythropoietin (e.g., U.S. Patent No. 4,703,008, incorporated herein by reference), alpha-1-antitrypsin, calcitonin, growth hormone, insulin, low density lipoprotein, apolipoprotein E, IL-2 receptor and its antagonists, superoxide dismutase, immune response modifiers, parathyroid hormone, the interferons (IFN alpha, beta, or gamma), nerve growth factors, glucocerebrosidase, colony stimulating factor, interleukins (IL) 1 to 15, granulocyte colony stimulating factor (G-CSF), granulocyte, macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), adenosine deaminase, insulin-like growth factors (IGF-1 and IGF-2), megakaryocyte promoting ligand (MPL, or thrombopoietin), etc.

The invention also provides non-human mammals with functioning non-native liver, e.g., human, or native liver which expresses a desired gene product. The animals can be used as models for evaluating a wide variety of disease

processes and treatments. For example, the animal models can be used to as models of pathogenesis for infections, e.g., viral infections such as human hepatitis viruses A, B and C, CMV, or the like, or to determine the effectiveness and safety of treatments or vaccines for such infections. The animals also find use for evaluating the treatment and prevention of genetic disorders, e.g., coagulation factor disorders, glycogen storage disease, alpha-1-antitrypsin deficiency, etc.

In one embodiment the non-human animals of the present invention contain a transgene which encodes a modified non-secreted uPA as described herein, e.g., uPA having a modified C-terminus containing KDEL, uPA having the signal peptide on the N-terminus substituted by the RR retention signal and transmembrane region of the type II transmembrane proteins (Schutze et al., EMBO J. 13: 1696-1705 (1994); Gorlich et al., Nature 357: 47-52 (1992), or a combination of both C-terminal and N-terminal modifications designed to inhibit secretion of the uPA molecule without substantially adversely affecting hepatotoxic activity. Expression of the modified uPA can be under the control of an inducible or constitutive promoter, e.g., the cytochrome P450 promoter of Jones et al., Nucl. Acids Res. Simultaneous with or subsequent to expression of the secretion impaired uPA transgene, non-native (e.g., human) hepatocytes are implanted in the transgenic mammal, e.g., a nude or immunodeficient *scid* mouse, to reconstitute the mammal's liver with a large proportion of non-native (e.g., human) hepatocytes. The mammal is then used as a model for ex vivo hepatic gene transfer, or it can serve as a model, for example, of human hepatitis C infection and its treatment, e.g., with ribozymes against HCV RNA as described herein.

In another embodiment the mammal can be transduced with an adenoviral vector encoding the modified, secretion-impaired uPA and the non-native cells implanted. The mammal's liver is reconstituted with the non-native hepatocytes and the animal used as described above.

The present invention also provides compositions and methods for treating or preventing hepatitis C infection using

therapeutic ribozymes. The ribozymes can be administered in a variety of ways, including by gene therapy targeted to the liver of an individual exposed to or infected by HCV.

5 A ribozyme of the invention targets the HCV RNA genome and RNA transcripts and copies thereof. Each ribozyme molecule contains a catalytically active segment capable of cleaving the plus or minus strand of HCV RNA, and further comprises flanking sequences having a nucleotide sequence complementary to portions of the HCV RNA. The flanking  
10 sequences serve to anneal the ribozyme to the RNA in a site-specific manner. Absolute complementarity of the flanking sequences to the target HCV sequence is not necessary, however, as only an amount of complementarity sufficient to form a duplex with the target RNA and to allow the  
15 catalytically active segment of the ribozyme to cleave at the target sites is necessary. Thus, only sufficient complementarity to permit the ribozyme to be hybridizable with the target RNA is required.

20 As used herein, the term "ribozyme" means an RNA molecule having an enzymatic activity that is able to cleave or splice other separate RNA molecules in a nucleotide base sequence specific manner. By reference to catalytic or enzymatic RNA molecule is meant an RNA molecule which has complementarity in a substrate binding region to a specific  
25 HCV RNA plus or minus strand target, and also has enzymatic activity that is active to cleave and/or splice RNA in that target, thereby altering the target molecule. By reference to HCV plus strand is meant one having the same polarity as viral mRNA and containing codon sequences that can be translated  
30 into viral protein. The minus strand is a noncoding strand that must be copied by an RNA-dependent polymerase to produce a translatable mRNA.

In preferred embodiments of the present invention the enzymatic RNA molecule is formed in a hammerhead motif,  
5 but the ribozyme may also be formed in the motif of a hairpin, hepatitis delta virus, group I intron or RNase P RNA (in association with an RNA guide sequence). Examples of hammerhead motifs are described by Rossi et al., AIDS Res.

Hum. Retrovir. 8: 183 (1992), hairpin motifs are described by Hampel et al., Biochem. 28:4929 (1989) and Hampel et al., Nucl. Acids Res. 18: 299 (1990), the hepatitis delta virus motif is exemplified in Perrotta and Been, Biochem. 31: 16 (1992), an RNaseP motif is described in Guerrier-Takada et al., Cell 35:849 (1983), and examples of the group I intron motif are described in Cech et al., U.S. Patent 4,987,071, each of the foregoing disclosures being incorporated herein by reference. These specific motifs are not limiting in the present invention and those of skill in the art will recognize that an enzymatic RNA molecule of the invention has a specific substrate binding site which is complementary to one or more of the target HCV RNA regions and that it has nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule.

The flanking sequences upstream and downstream of the ribozyme catalytic site may comprise segments of any length that effectively imparts the desired degree of targeting specificity for the ribozyme. Preferably a flanking sequence comprises from about 4 to about 24 nucleotides, more preferably from about 6 to about 15 nucleotides, and typically about 9 to 12, and results in base pairing to the substrate sequence immediately upstream and downstream of the plus or minus strand HCV RNA sequences which comprise the cleavage site.

To select therapeutically useful ribozymes specific for HCV RNA, ribozymes are selected and expressed in whole cells. An optimized expression cassette for the ribozyme can be used where the sequence is embedded in a stable loop region which, in turn, is part of an adenoviral *va* RNA, so that a catalytic secondary structure can form independently from the surrounding RNA of the expressed RNA. A library of ribozymes flanked by random sequences are cloned into the loop region of the expression cassette. Ribozymes are selected from the library using the HCV RNA target sequence itself. Using this strategy a ribozyme is selected for cleavage sites that are accessible within target HCV RNA and that have structures that permit efficient cleavage. Ribozymes are selected against

conserved regions of the HCV genome to be effective against as many HCV strains as possible and to reduce the opportunity for escape variants. These regions include the plus or minus strands of the 5' noncoding region and those coding for the core protein and NS5 RNA polymerase. To confirm the effectiveness of the ribozymes in mammalian cells, HCV cDNA is transduced into hepatoma cells to produce intact HCV RNA. Cells which stably produce high level expression of HCV RNA are selected and then transfected with the ribozyme expression cassette. The resulting cleavage of HCV RNA by a ribozyme produces a 5'OH group and a 2'-3' cyclic phosphate group, thereby creating an unstable molecule and decreasing the HCV mRNA concentration within the cell. Ribozyme producing cell lines are compared for the production of HCV RNA and those ribozymes with optimal activity are selected. Cross-reactivity of the ribozyme with different HCV types can also be determined in this system. Ribozymes directed against different target sites in a particular mRNA can be simultaneously isolated using this procedure. This approach has permitted the selection of ribozymes against HCV RNA which completely eliminate HCV RNA expressed in CHO cells.

The site of cleavage in a target HCV RNA molecule is also dependent on the type of ribozyme, e.g., when the ribozyme is of the hammerhead type, the substrate cleavage site is immediately 3' to the sequence NUH, where N is any nucleotide, U is uridine, and H is any nucleotide except G. Different types of ribozymes can be used to achieve the specific cleavage of the targeted HCV RNA molecule, e.g., different hammerhead ribozymes (at least 14 different members of this class), the larger Group I introns, RNase P (which targets tRNA), hairpin ribozymes, hepatitis delta virus ribozyme, etc.

As mentioned above, the HCV RNA target region is typically one that is substantially conserved among the prevalent strains of HCV. These regions include the 5' noncoding region, the core protein, and the nonstructural proteins NS-2, NS-3 (helicase), NS-4, NS-5 (RNA polymerase), and conserved regions of E1 (gp30) and NS-1 (gp72).

Representative examples of HCV ribozyme target sequences include, for HCV types 1a and 1b (where putative cleavage sites are indicated by a "-"), ribozyme 1 (Rz1):

CGGGAGGTCTCGTA [Seq. ID:1] (5' UTR, nucleotides 317 to 332;  
5 plus strand), Rz2: GCACCATGAGCACGAA [Seq. ID:2] (nucleotide  
337 to 352; minus strand), Rz5: CTAAACCTCAAAGAAA [Seq. ID:3]  
(core, nucleotide 355 to 370; plus strand), Rz4:  
CCAACCGTCGCCCACAG [Seq. ID:4] (core, nucleotide 385 to 401;  
plus strand), Rz3: CCCACAGGACGTCAAG [Seq. ID:5] (core,  
10 nucleotide 385 to 410; minus strand), and Rz6:  
GGTAAGGTCATCGATA [Seq. ID:6] (core, nucleotide 699 to 714;  
plus strand).

A sequence comprising or encoding said ribozyme or a combination of ribozymes targeted to different portions of the  
15 HCV RNA can be delivered in a wide variety of ways to HCV-infected or HCV-susceptible cells to interrupt or prevent HCV infection. The ribozyme can be administered as RNA or expressed from an expression vector. The ribozyme can be administered ex vivo, i.e., contacted with cells that have  
20 been removed from an infected individual, treated and returned, or the ribozyme can be administered in vivo. Delivery can be via an appropriate delivery vehicle, e.g., a liposome, a controlled release vehicle, by use of iontophoresis, electroporation or ion paired molecules, or  
25 covalently attached adducts, and other pharmacologically acceptable methods of delivery. Preferably a carrier provides a means to accumulate the ribozyme at the primary site of HCV infection, i.e., the liver. The ribozyme delivery vehicle can be designed to serve as a slow release reservoir or to deliver  
30 its contents directly to the target cell. WO 94/16736 describes a process for evolving RNA molecules to bind receptors on liver cells, to which RNA a ribozyme of the present invention may be tethered for targeting purposes. Examples of ribozyme delivery vehicles include liposomes,  
35 hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Liposomes can readily be targeted to the liver for delivery of RNA to infected hepatocytes. In a preferred embodiment the anti-HCV ribozyme is administered

via an expression vector that is suitable for delivery and expression of an oligonucleotide comprising said ribozyme in a mammalian host cell.

Routes of ribozyme administration include

5 intramuscular, aerosol, intravenous, parenteral, intraperitoneal, etc. The specific delivery route for a selected ribozyme will depend on a variety of factors, such as the form of the ribozyme, the intended target, the stage of disease, etc. For example, while unmodified ribozyme is taken  
10 up by cells, modifications can be made to enhance cellular uptake, e.g., by reducing the ribozyme's charge to produce a molecule which is able to diffuse across the cell membrane. The structural requirements necessary to maintain ribozyme catalytic activity are generally recognized in the art, as  
15 described in, e.g., Cech, Curr. Op. Structural Biol. (1992), which is incorporated herein by reference. Ribozyme modifications to enhance cellular delivery can also be designed to reduce susceptibility to nuclease degradation.

The dosage of ribozyme will also depend on a variety  
20 of factors, such as the form of the ribozyme, the route of administration, the severity of infection or stage of disease, the general condition of the patient being treated, and thus can vary widely. Generally the dosage of ribozyme will be between about 10  $\mu$ g-200 mg/kg of body weight per day and  
25 result in therapeutic or prophylactic levels within the targeted cells sufficient to inhibit or eradicate HCV from the cells. Establishment of therapeutic or prophylactic levels of ribozyme within an HCV-infected cell depends upon, e.g., the rate of uptake (or expression by a particular vector), and  
30 rate at which the ribozyme is degraded. The duration of treatment may extend throughout the course of HCV infection or disease symptoms, usually at least about 7-30 days, with longer durations being necessary for severe infections. The number and timing of doses can vary considerably, depending  
35 on, e.g., the extent of infection, the efficacy of a particular ribozyme or mixture thereof, the delivery vehicle and route of administration, the judgment of the prescribing physician, etc. As used herein, the terms "treatment" or

"treating" refer to any ribozyme-based treatment of HCV or related disease, and include: (1) preventing HCV disease from occurring in a subject who does not have the disease or who has not yet been diagnosed as having it, including prophylactic uses to individuals susceptible to or suspected of exposure to HCV; (2) eradicating, inhibiting or arresting the development of HCV infection or related disease; or (3) regressing or reversing the disease.

The ribozymes of the invention may be prepared by chemical synthesis or produced by recombinant vectors according to methods established for the synthesis of RNA molecules. See, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), incorporated herein by reference. The ribozyme sequence may be synthesized, for example, using RNA polymerases such as T7 or SP6. The ribozymes of the invention may be prepared from a corresponding DNA sequence (DNA which on transcription yields a ribozyme) operably linked to an RNA polymerase promoter such as the promoter for T7 RNA polymerase or SP6 RNA polymerase. A DNA sequence corresponding to a ribozyme of the present invention may be ligated into a DNA vector, such as a plasmid, bacteriophage or other virus. Where the transfer vector contains an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, the ribozyme may be conveniently produced upon incubation with an RNA polymerase. Ribozymes may therefore be produced in vitro by incubation of RNA polymerase with an RNA polymerase promoter operably linked to DNA corresponding to a ribozyme, in the presence of ribonucleotides. In vivo, procaryotic or eucaryotic cells (including mammalian cells) may be transfected with an appropriate vector containing genetic material corresponding to a ribozyme in accordance with the present invention, operably linked to an RNA polymerase promoter such that the ribozyme is transcribed in the host cell. Ribozymes may be directly transcribed in vivo from a transfer vector, or alternatively, may be transcribed as part of a larger RNA molecule. For example, DNA corresponding to ribozyme



sequences may be ligated into the 3' end of a carrier gene, for example, after a translation stop signal. Larger RNA molecules may help to stabilize the ribozyme molecules against nuclease digestion within cells. On translation the carrier gene may give rise to a protein, whose presence can be directly assayed if desired, for example, by enzymatic reaction when the carrier gene encodes an enzyme.

In one exemplary method of producing a ribozyme, two synthetic oligonucleotides of complementary sequence are prepared by standard procedures on an automated DNA synthesizer and hybridized together, where one of the oligonucleotides encodes a desired ribozyme. The respective ends of the hybridized oligonucleotides correspond to different restriction enzyme sites and, after appropriate cleavage, the double stranded DNA fragment is cloned into a transfer vector. Where the plasmid vector contains a RNA polymerase promoter upstream from the DNA sequence corresponding to a ribozyme of the present invention, RNA transcripts corresponding to the ribozyme are conveniently prepared either in vitro or in vivo. When in vivo, that is, within the cell or cells of an organism, a transfer vector such as a bacterial plasmid or viral RNA or DNA encoding one or more ribozymes may replicate and/or be transcribed by cellular polymerases to produce ribozyme RNAs which then inactivate a desired target HCV RNA sequence. The transfer vector may become integrated into the genome of the host cell and transcription of the integrated genetic material gives rise to ribozymes which act to inactivate the target HCV RNA.

Accordingly, a viral vector containing a sequence corresponding to a ribozyme of the present invention can be prepared in any of a wide variety of ways. Representative retroviral vectors suitable for use in the present invention are described above. Other vectors may also be employed, particularly for the ex vivo methods described herein, such as DNA vectors, pseudotype retroviral vectors, adenovirus, adeno-associated virus, gibbon ape leukemia vector, VSV-G (e.g., as described in WO 94/29440), VL30 vectors, liposome mediated vectors, and the like.

Because adenovirus is capable of infecting dividing and non-dividing hepatocytes at high efficiency, in a preferred embodiment the vector is an adenovirus (or adeno-associated virus vector). Representative adenoviral vectors which can be used to encode the ribozymes of the present invention are described in Stratford-Perricaudet et al., supra, Graham and Prevec, supra, Barr et al., supra, WO 94/20146, WO 94/26915, and WO94/29471, and adeno-associated vectors are described in U.S. 5,436,146, each of which is incorporated herein by reference. A preferred adenovirus plasmid for producing recombinant adenovirus which drives transcription of a ribozyme of the invention is the pXCJL.1 plasmid described in Spessot et al., Virology 168:378-387 (1989), incorporated herein by reference. An adenoviral vector may include essentially the complete adenoviral genome (Shenk et al., Curr. Top. Microbiol. Immunol. 111: 1-39 (1984) or may be a modified adenoviral vector in which at least a portion of the adenoviral genome has been deleted. In a preferred embodiment, the adenoviral vector comprises an adenoviral 5' LTR, an adenoviral 3' ITR, an adenoviral encapsidation signal; at least one DNA sequence encoding a ribozyme of the present invention; and a promoter controlling the transcription of the ribozyme or an RNA polymerase and a promoter controlling the transcription of the RNA polymerase sequence, e.g., T7 polymerase controlled by the PGK promoter. The vector is typically free of at least the majority of adenoviral E1, E2 and E4 DNA sequences.

The vector is preferably packaged into infectious, nonreplicating, recombinant adenoviral particles using, e.g., a helper adenovirus or plasmid (e.g., pGM17, or pBHG10 as described in Bett et al., Proc. Natl. Acad. Sci. USA 91:8802-8806 (1994), incorporated herein by reference), or cell line which provides the necessary encapsidation materials. Preferably the helper virus has a defective encapsidation signal so the helper virus will not encapsidate itself. An example of an encapsidation defective helper virus which may be employed is described in Grable et al., J. Virol. 66:723-731 (1992), incorporated herein by reference.

The vector and the encapsidation defective helper virus are transfected into an appropriate cell line for the generation of infectious viral particles. Transfection may take place by electroporation, calcium phosphate precipitation, microinjection, or through proteoliposomes. Examples of appropriate cell lines include, but are not limited to HeLa cells or 293 (embryonic kidney epithelial) cells (ATCC No. CRL 1573). The infectious viral particles (i.e., the adenoviral vector) may then be transduced into eucaryotic cells, such as hepatocytes, whereby the DNA sequence encoding a ribozyme (or other molecule, as desired) is expressed by the eucaryotic cells in a host.

The viral vector, consisting of infectious, but replication-defective, viral particles, which contain at least one DNA sequence encoding a ribozyme effective against HCV RNA, is administered in an amount effective to inhibit or prevent HCV infection in a host. The vector particles may be administered in an amount from 1 plaque forming unit to about  $10^{14}$  plaque forming units, more preferably from about  $1 \times 10^6$  plaque forming units to about  $1 \times 10^{13}$  plaque forming units. A sufficient number of adenoviral vector particles containing a sequence corresponding to a ribozyme of the invention should be administered to the liver to infect up to at least about 50% of the hepatocytes, usually about 80%, preferably about 95%, and more preferably 99% to 99.99% or more of the hepatocytes in the individual, e.g., typically from about 10 up to about 100 or more adenovirus particles per hepatocyte are administered. The host may be a human or non-human animal host. A preferred non-human animal host is a mammal, more preferably a non-human primate or a non-human mammal having a liver which is comprised at least partially of human hepatocytes, as more fully described herein. The adenovirus vector can be administered by a variety of routes, but typically systemically, such as, for example, by intravenous administration (e.g., peripheral vein injection), by infusion via the portal vein, to the bile duct, intramuscularly, intraperitoneally, or intranasally.

The expression of the ribozyme sequence encoded by the vector can be constitutive or inducible, but preferably is constitutive. As the hepatocytes which express the ribozyme inhibit the proliferation of and are capable of eradicating HCV, virus-free hepatocytes may repopulate the liver. Thus, over a period of days to weeks hepatocytes treated with the anti-HCV ribozyme may repopulate the liver. The entire treatment process itself may be repeated as necessary, understanding that a humoral immune response to certain vectors, e.g., adenovirus vector, may be generated by repeated administration. The immune response may ameliorate the effect of vector administration and thus necessitate administration of larger quantities of the vector, administration of a different vector to which the patient is not immune, delivery of the vector in a manner by which the particles are shielded from the host's immune system, or tolerization of the host's immune system to the vector.

The vector encoding the anti-HCV ribozyme is also used to transduce hepatocytes which have been isolated from a patient. For ex vivo transduction the viral vector is preferably a retroviral vector, although other vectors may also be used. In the case of liver transplants, the liver may be transduced with the vector, e.g., adenovirus, prophylactically prior to transplant, while in the donor or ex vivo, or after transplant but before HCV infection of the donor liver is substantially established.

In an animal model of HCV disease and treatment, the sequences encoding the selected ribozymes are placed into adenoviral vectors and used to transduce the hepatocytes of the animal of interest, e.g., mice in which the liver has been ablated with the urokinase gene as described herein and reconstituted with human hepatocytes. For example, scid mice that have livers reconstituted with human hepatocytes are infused with hepatitis C particles, or human HCV-infected hepatocytes are used in the reconstitution process. The liver and serum of the animals are monitored for production of virus by quantitative RT-PCR assays. Additionally, immunohistochemical staining of tissues or antigen detection

in the blood can be performed. The ribozyme-expressing adenovirus is delivered to the animal and efficacy of HCV inhibition observed.

The following Examples are offered by way of illustration, not limitation.

#### EXAMPLE I

To induce liver regeneration, a recombinant adenoviral vector that expresses human urokinase from the RSV-LTR promoter, Ad.RSV-uPA was constructed. Human uPA can activate plasminogen across species (Wohl et al., Biochim. Biophys. Acta. 745:20-31 (1983)).

For construction and production of the recombinant adenoviral vectors, the cDNA for human uPA was obtained from B. Hinzmann (MDC, Berlin) and is described in Nagai et al., Gene, 36:183-188 (1985), incorporated herein by reference). The 1.326 kb HindIII/Asp718 uPA fragment that contains the protein coding sequence was inserted into the HindIII/Asp718 sites of pXCJL.1 (Spessot et al., Virology 168:378-387 (1989)) under the transcriptional control of the Rous Sarcoma Virus LTR (RSV) promoter, and upstream of the bovine growth hormone polyadenylation signal (Kay et al., Proc. Natl. Acad. Sci. USA 91:2353-2357 (1994)). The virus was prepared after co-transfection with pJMI7 (McGrory et al., Virology 163:614-617 (1987)) and the vector designated Ad.RSV-uPA.

The screening for Ad.RSV-uPA was carried out by amplification of individual plaques in 293 cells. Three days after infection the supernatant was tested for immunological reactive uPA by ELISA and fibrinolytic activity by fibrin plaque assay (Jespersen et al., Haemostasis 13:301-315 (1983)). The last test demonstrated the catalytic activity of uPA produced after Ad.RSVuPA infection.

The construction of viral vector Ad/RSV-hAAT is described in Kay et al., Hepatology 21: 815-819 (1995), and the construction of the vector Ad.RSV $\beta$ Gal is described in Stratford-Perricaudet et al., J. Clin. Invest. 90:626-630 (1992), incorporated herein by reference. The recombinant

viruses were prepared and purified as described in Barr et al., Gene Therapy 2:151-155 (1995), incorporated herein by reference. The purified virus was stored in aliquots at -80°C and freshly diluted with HgDMEM media prior to injection. The viral titers were determined by OD measurements and plaque assay (Graham et al., Methods in Molecular Biology: Gene transfer and expression protocols 7:109-128, The Humana Press, (1991)) and ranged from 2 to 5 x 10<sup>11</sup> pfu/ml. All viral preparations tested negative for the presence of replication competent virus by an assay that can detect 1 wild-type particle per 5 x 10<sup>5</sup> pfu of E1 deleted virus (Barr et al., supra).

The construction of the retroviral vectors LTR-cFIX (Kay et al., Science 262:117-119 (1993)), LNAIbhAAT and LBgeo (Kay et al., Hum. Gene Ther. 3:641-647 (1992)), each of which is incorporated herein by reference, has been described. Recent characterization of the LNAIbhAAT vector showed that a portion of the albumin promoter has been deleted and that a 0.8kb promoter-enhancer fragment remained in the vector. The viruses were titered on 208F cells and the titer for LBgeo (Kay et al., Hum. Gene Ther. 3:641-647 (1992)) was 1 x 10<sup>6</sup> cfu/ml while the titers for LTR-cFIX (Kay et al., Science 262:117-119 (1993)) and LNAIbhAAT were 2 x 10<sup>6</sup> cfu/ml. The titer of LNAIbhAAT was about 10-fold less than the original clone used (Kay et al., Hum. Gene Ther. 3:641-647 (1992)). Retroviral packaging cell lines were maintained in high glucose DMEM with 10% bovine calf serum (Hyclone). Virus was harvested from confluent packaging lines (in 10 cm dishes) 16 hrs after medium change (5 ml), filtered through a 0.45 µm filter, mixed with polybrene (12 µg/ml) and used immediately for infusion in animals.

C57BL/6 female mice aged 5 to 6 weeks (Jackson Laboratories, Bar Harbor, Maine) were housed in a specific pathogen free environment. Blood samples were obtained by retroorbital bleeding. For portal vein cannulation, mice were anesthetized by an intraperitoneal administration of 0.5 ml of 20 mg/ml 2,2,2-Tribromoethanol. A midline abdominal incision was made and the skin was separated from the peritoneum to

create a subcutaneous pocket. The peritoneum was opened and the portal vein was exposed. A silicone tube (0.02" I.D., 0.037" O.D., S/P Medical Grade, Baxter, IL) was inserted in the portal vein and perfused with heparinized saline (1u/ml).  
5 An adhesive (Histoacryl Blau, Braun Melsungen AG, Germany) was used to fix the cannula. Thereafter the cannula was tunneled through the peritoneum and secured with a 4.0 silk suture. The 3 cm long cannula was tied off at the distal end and placed subcutaneously in the previously created pocket.

10 The mice were given the virus no earlier than 24 hrs later. In some mice the portal vein cannulation was performed together with a 2/3 hepatectomy. The partial hepatectomy was carried out according to the procedure described in Kay et al., Hum. Gene Ther. 3:641-647 (1992), incorporated herein by  
15 reference.

To perfuse the portal vein, mice were anesthetized, the skin was opened at the proximal site of the already existing abdominal incision. The cannula was exposed and connected to a syringe pump. For virus infusion,  $0.5 \times 10^{10}$   
20 or  $1.0 \times 10^{10}$  pfu of adenovirus (Ad.RSV-uPA, Ad/RSV-hAAT, Ad.RSV- $\beta$ Gal) in 150  $\mu$ l DMEM were injected over 5 to 10 min into the portal vein through the cannula. Retrovirus perfusion was performed either 48 hr after partial hepatectomy or at different time points (days 3, 5, 7, 9) after adenovirus  
25 administration. One ml of filtered retrovirus supernatant with 12  $\mu$ g/ml of polybrene was infused into the portal vein via the cannula over 50 min with a syringe pump.

All biochemical and histological analysis were performed after injection of  $0.5 \times 10^{10}$  pfu adenovirus into  
30 the portal vein through the cannula. The ELISA assay for uPA was based on two different monoclonal antibodies directed against the catalytic and receptor-binding domain of uPA. One of the monoclonal antibodies was labelled with peroxidase according to the manufacturer's (Pierce) specifications. The  
5 ELISA had a linear range from 1 ng/ml - 50 ng/ml. Human  $\alpha$ 1 antitrypsin (Kay et al., Hum. Gene Ther. 3:641-647 (1992)) and cFIX (Kay et al., Science 262:117-119 (1993)) concentrations in serum samples were determined by ELISA assay

as described. The assays had a linear detection range from 2 to 100 ng/ml. A SIGMA diagnostic kit was used for colorimetric determination of the activity of serum glutamic pyruvic transaminase (SGPT) using 10  $\mu$ l of serum according to the protocol of the manufacturer (SIGMA procedure: No. 505). Serum total protein and albumin were analyzed by routine automated methods in the clinical pathology laboratories of the University of Washington Hospital, Seattle, WA. The prothrombin time was determined by a SIGMA Diagnostic kit (Procedure: No. T7280). The test was carried out with 100  $\mu$ l plasma obtained from citrated blood. The PTs were measured in the presence or absence of 20  $\mu$ g/ml aprotinin. For  $^3$ H-thymidine incorporation, the method of Paulsen and Reichelt (Paulsen et al., Virchow Archiv B Cell Pathology 62:173-177) was modified. One  $\mu$ Ci of 6- $^3$ H-Thymidine (5 Ci/mmol, Amersham) in 0.9% saline per gram body weight was injected intraperitoneally 3 times: 24h, 12h and 45 min prior to sacrificing the animals. One-third of the liver was fixed in 10% neutral formalin for histological autoradiography. 0.3g of liver was homogenized in 5ml 0.2N perchloric acid in a glass Dounce homogenizer on ice and then centrifuged. The pellet was washed once in 5 ml cold 0.2N perchloric acid, once with 5 ml ethanol/ether (1/1, v/v) and once with 2 ml ethanol to remove the acid soluble nucleotides. DNA in the pellet was then hydrolyzed in 5 ml 0.5N perchloric acid at 90°C for 10 min. Following centrifugation, duplicate aliquots of hydrolyzed DNA were determined for radioactivity. The DNA content in the supernatant was determined by OD reading and by the diphenylamine method (Bucher, Int. Rev. Cytol. 15:245-278 (1963)). Specific activity was expressed as cpm/ $\mu$ g DNA.

Infusion of  $1 \times 10^{10}$  adenoviral pfu into the portal vein of C57BI/6 mice was known to result in transduction of 100% of hepatocytes with more than 1 copy of adenoviral DNA per cell (Li et al., Human Gene Ther. 4:403-409 (1993)). The same dose of Ad.RSV-uPA resulted in 90% mortality that at least in part was related to hemorrhage. When  $5 \times 10^9$  pfu of Ad.RSV-uPA was used, the mortality rate was less than 5% and this dose was selected for the majority of the liver



regeneration experiments. For comparison, control studies were performed on mice that received the Ad/RSV-hAAT virus or 2/3 partial hepatectomy. After infusion of adenovirus or partial hepatectomy, blood samples were analyzed at different times for serum urokinase concentrations, SGPT (an indicator of hepatocyte damage) and prothrombin time (PT).

The infusion of Ad.RSV-uPA resulted in transient elevations of serum urokinase reaching a peak value of 350 ng/ml (70 to 100 times greater than endogenous levels) four days later before falling to background concentrations by day 12 (Fig. 1A). The rise in uPA was associated with an increase in the serum SGPT concentrations reaching 800 u/ml on day 4 (nl range 20 to 40 u/ml) before slowly falling to normal levels between days 12 to 13. No or minimal elevation in serum SGPT (less than 2-fold greater than normal) was found in Ad/RSV-hAAT treated animals.

At varying times after adenovirus infusion, animals were infused with <sup>3</sup>H-thymidine, and the amount of radioactivity incorporated into liver DNA was determined as a means to quantitate cell proliferation (Fig. 1B). In agreement with previous studies (Grundmann et al., in Liver Regeneration After Experimental Injury, Lesch & Reuter (eds), Stratton Intercontinental Medical Book Co., NY (1973)), the partial hepatectomized animals had a sharp peak of <sup>3</sup>H-thymidine uptake 48 hours postoperatively. However, the animals treated with Ad.RSV-uPA had an increased period of <sup>3</sup>H-thymidine uptake that began on day 3 and persisted for 8 days. Thus, the period of hepatic <sup>3</sup>H-thymidine uptake with Ad.RSV-uPA treatment was much greater than that obtained with partial hepatectomy. The recipients of the Ad/RSV-hAAT control adenovirus had a peak of hepatic <sup>3</sup>H-thymidine uptake on day 4 that returned to baseline levels 24h later and a minimal rise in <sup>3</sup>H-thymidine uptake on day 11. In total, the hepatic damage as measured by SGPT levels and high rates of <sup>3</sup>H-thymidine uptake was attributed to intrahepatic urokinase production.

The PT measured as a means to estimate the coagulation status in Ad.RSV-uPA recipient animals reached

more than 200 seconds on day 3 but fell to the normal range between days 11 and 13 (Fig. 1A). Animals which received a partial hepatectomy or control adenovirus had normal PTs. The observed disorders in clotting function in Ad.RSV-uPA transduced mice may have resulted from the elevated serum uPA activity or decreased synthesis of clotting factors by the liver due to hepatocyte degeneration and liver insufficiency. To distinguish between these two possibilities, the PTs were compared in the presence and absence of aprotinin, a plasmin inhibitor. Whereas the PT without aprotinin was 10 times higher than normal (220 sec on day 3) (Fig. 1A), the PT value in the presence of aprotinin was almost normal (35 sec). The high PT without aprotinin likely resulted from active urokinase in the plasma sample that continued to convert plasminogen to active plasmin (and delete fibrin strand formation) *in vitro*. Thus, Ad.RSV-uPA treated mice did not have a deficiency in clotting factors. Additionally, these animals had normal serum albumin and total protein concentrations. The presence of functional hepatic clotting factors and normal concentrations of serum proteins suggested that significant liver biosynthetic function continued to occur after urokinase induced hepatocellular damage.

For histological analysis liver samples were fixed in 10% neutral formalin, embedded in paraffin and stained with hematoxylin/eosin. The livers of animals that received  $1 \times 10^{10}$  pfu of Ad.RSV-uPA (but not Ad/RSV-hAAT) had a white appearance which was observed on days 3 to 7 after adenovirus administration and was similar in gross morphology to that observed in the urokinase transgenic mouse model (Sandgren et al., Cell 66:245-256 (1991)). Animals receiving half the dose of adenovirus had an intermediate appearing whitish red liver that was most pronounced around the portal areas. Fourteen days after virus administration the livers appeared normal.

Microscopic histological findings from animals treated with  $5 \times 10^9$  pfu of recombinant adenovirus indicated that by day 3, Ad.RSVuPA treated mice had a moderate inflammatory infiltrate that contained macrophages and neutrophils. Degenerative changes in hepatocytes included

vacuolization, pyknotic and few mitotic nuclei. By day 4 there was widespread hepatocyte degeneration in about 90% of the hepatocytes. Eight to 10 days after Ad.RSV-uPA administration there was evidence of hepatic recovery including the presence of multifocal regeneration (mostly in the periportal spaces), heterogenous size of nuclei, and a much decreased inflammatory reaction with few degenerating hepatocytes. By three to four weeks, the infiltrate had resolved and the liver appeared normal.

In contrast, the animals receiving the control Ad.RSV-hAAT adenovirus had a mild infiltrate mostly localized to the periportal spaces without evidence of hepatic necrosis and degeneration on days 3 to 6. On days 7 to 9 the liver parenchyma appeared normal but a secondary mild infiltrate was observed on days 11-13 that resolved by day 14. Inflammatory reactions have been observed with adenovirus administration which have been associated with an immunologic response directed against adenoviral transduced cells. This is believed to result from low level production of adenovirus antigens in transduced cells (Yang et al., Proc. Natl. Acad. Sci. USA 91:4407-4411 (1994)).

To definitively establish that hepatocytes regenerated after Ad.RSV-uPA administration, autoradiography was performed on liver sections obtained from the animals described for Fig. 1. Animals received thymidine injections as described above. Six  $\mu$ m sections were dip-coated with Kodak NTB-2 emulsion diluted 1/1 (v/v) with water, and developed after a 2 week exposure. All slides were counterstained with hematoxylin/eosin. More than 50% of the hepatocyte nuclei from Ad.RSV-uPA treated mice were observed with 3H-label over the period of days 3 to 11 post transduction. The asynchronous DNA replication in different hepatocytes forms the characteristic incorporation curve in Fig. 1B. A few degenerating hepatocytes and non-parenchymal cells were labelled in these sections. The frequency of hepatocyte labelling was about 80% in partial hepatectomized control animals 48 hrs post operatively.

In contrast, the majority of radioactive labelling in Ad/RSV-hAAT treated mice were observed in the inflammatory cells, most of which were localized around the periportal region. These labelled cells were present on days 4 and 11 but were not present in appreciable quantities in between these two time points. Because 3H-thymidine was infused over a 1 day period, it is possible that the inflammatory cells were labelled prior to taking residence in the liver. The presence of labelled inflammatory cells correlated with the amount of radioactive DNA in the liver (Fig. 1). Hepatocyte labelling was detected in the Ad/RSV-hAAT control animals on day 4 but at much reduced levels compared with partial hepatectomized or Ad.RSV-uPA treated animals. Normal untreated mice had rare labelled nuclei. In total, these studies demonstrate that urokinase expression from hepatocytes induced significant liver parenchymal cell regeneration that lasted for a period of 8 days.

The livers from Ad.RSV-uPA treated animals were analyzed for alphafetoprotein (AFP), an oncodevelopmental protein, by immunohistochemical staining. Deparaffinized sections were incubated with a 1:10 dilution of polyclonal rabbit anti-human alpha-fetoprotein antibodies (DAKO) which crossreact with mouse AFP. Specific binding was detected by horseradish peroxidase conjugated goat anti-rabbit antibodies. Human fetal liver was used as a positive control. The results demonstrated that while rare degenerating hepatocytes contained AFP, none of the newly regenerating cells expressed AFP. This indicates that like partial hepatectomy, uPA-induced regeneration occurs by division of mature hepatocytes without dedifferentiation (Fausto, Curr. Opinion Cell Biol. 2:1036-1042 (1990)).

#### EXAMPLE II

##### Ad.RSV-uPA Treatment Results in Permanent Retroviral-Mediated Gene Transfer In Vivo

This Example demonstrates retroviral-mediated gene transfer into the uPA induced regenerating hepatocytes.

Recombinant retrovirus that contained the human  $\alpha 1$  antitrypsin cDNA (LNAlbHAAT) was infused (1 ml of  $2 \times 10^6$  pfu) into the portal vein of mice at different times after Ad.RSV-uPA administration. Some mice received multiple

5 infusions of retrovirus. The control animals underwent a partial hepatectomy or were infused with the Ad.RSV- $\beta$ gal adenovirus in place of Ad.RSV-uPA. The quantity of gene product and the relative hepatic gene transfer rate was determined by periodic measurements of serum HAAT (Fig. 2).

10 Partial hepatectomized animals that were injected with LNAlbHAAT retrovirus had constitutive HAAT serum concentrations that varied between 20 to 120 ng/ml in five out of the first six recipients (Fig. 2A). One animal had an unusually high persistent serum HAAT concentration of about

15 400 ng/ml after partial hepatectomy. To determine whether the high rate of gene transfer observed with partial hepatectomy in the one mouse could be reproduced, six additional mice were treated in a similar manner and all of these animals had serum HAAT concentrations in the 20 to 100 ng/ml range. For partial

20 hepatectomized mice, retroviral-mediated gene transfer only occurred during a small window at about 48 hours (Kay et al., Hum. Gene Ther. 3:641-647 (1992)) and multiple infusions of retrovirus after partial hepatectomy did not increase gene transfer.

25 The mice that were infused with a single dose of LNAlbHAAT retrovirus 3 days after Ad.RSV-uPA administration had a high peak of serum HAAT (up to 800 ng/ml) that fell during the first 10 days to constitutive levels ranging from 10 to 100 ng/ml in individual animals (Fig. 2B). The peak

30 level of serum HAAT may have been due to the continued hepatocyte turnover that occurred on days 3 and 4 after Ad.RSV-uPA administration.

The mice that received three retrovirus (LNAlbHAAT) infusions on days 3, 5 and 7 after Ad.RSV-uPA had constitutive

35 HAAT levels in the 100 to 400 ng/ml range (Fig. 2C). Thus, gene expression as determined by the quantity of the HAAT serum marker was about 5-fold higher using the Ad.RSV-uPA mediated regeneration compared to that obtained with partial

hepatectomy. When the retrovirus was infused on days 5, 7 and 9 after Ad.RSV-upa administration, the serum HAAT levels ranged from 100 to 200 ng/m (Fig. 2D). The serum HAAT concentrations from mice that had been infused with a control adenovirus prior to LNAIbHAAT infusion(s) were similar to mice that had received a partial hepatectomy (Fig. 2E and F). The retrovirus transduction which occurred in mice that received the control adenovirus was likely related to the low level of hepatocyte regeneration. Multiple infusions of LNAIbHAAT after control adenovirus did not lead to an increase in serum concentrations of HAAT (Fig. 2F). Because gene expression was higher in animals given multiple injections, the improved rate of hepatic gene transfer was likely due to the ability to infuse multiple doses of retrovirus.

A second recombinant retroviral vector, LTR-cFIX that expresses a therapeutic protein, canine factor IX (Kay et al., Science 262:117-119 (1993)) was used to corroborate the findings described above. The data presented in Fig. 4B demonstrated that like HAAT, the serum canine factor IX concentration was several fold higher after Ad.RSV-uPA induced regeneration compared with partial hepatectomy (15-30 ng/ml vs 5-10 ng/ml). Although low, the concentration of cFIX obtained by prokinase induced liver regeneration was greater than that which was obtained in a dog model of hemophilia B (Kay et al., *ibid.*). The low absolute serum concentration of cFIX may be somewhat misleading because of potential for an altered biological half-life of the canine protein in mice. For example, the biological half-life of human factor VIII and factor IX is reduced by at least 6- and 3-fold respectively when infused in mice (Hoebe et al., Hum. Gene Ther. 4:179-186 (1993) and Smith et al., Nature Genetics 5:392-402 (1993)).

To establish that gene transfer resulted in hepatocyte transduction, mice that underwent either partial hepatectomy or Ad.RSV-uPA infusion were injected via the portal vein with the LBgeo retroviral vector that expresses E.coli beta-galactosidase. Three weeks later, a small portion of the liver was removed for histochemical X-gal staining. From the remaining liver, hepatocytes were isolated, cultured

and stained with X-gal. The proportion of blue cells were determined from the population of cultured hepatocytes. For histochemical  $\beta$ -galactosidase analysis, the method was modified as described (MacGregor et al., Humana Press Inc. (1989)). Histochemical analysis for beta-galactosidase activity was performed on thin sliced liver samples that were fixed for 60 min in 4% p-formaldehyde, 0.1M  $\text{NaH}_2\text{PO}_4$  pH7.3 at 4°C, then rinsed 3 times for 30 min with 0.1M  $\text{NaH}_2\text{PO}_4$ , 2mM  $\text{MgCl}_2$ , 0.01% Na-deoxycholate, 0.02% NP40 and stained overnight at 37°C with rinsing buffer containing 1 mg/ml X-Gal, 5mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 5mM  $\text{K}_4\text{Fe}(\text{CN})_6$  pH7.3. After 24h postfixation in 10% neutral formalin, the liver was embedded in paraffin, cut, and counterstained with hematoxylin/eosin.

Histochemical X-gal staining of liver sections from Ad.RSV-uPA and LBgeo treated mice, showed some single blue cells and clonal populations of blue cells of up to 6 blue cells per clone while the proportion of  $\beta$ gal positive hepatocytes in culture varied from 7 to 7.5%. This suggests that at least 2 to 3 cell divisions occurred from the time of retrovirus transduction. In partial hepatectomized animals, rare clones of 2 cells were detected and the proportion of  $\beta$ gal positive cells were 0.8%. The transduction efficiencies using the LBgeo vector were in agreement with the relative quantities of hAAT/cFIX using LNAIbhAAT/LTR-cFIX that were seen in Fig. 2 and confirm that retroviral-mediated gene transfer was greater using the urokinase-induced hepatic regeneration compared to partial hepatectomy.

Thus, this Example demonstrates that although this gene transfer strategy caused a transient hepatocellular injury, there was full recovery within a few weeks. Furthermore, there was no evidence of hepatobiosynthetic deficiencies after Ad.RSV-uPA administration. This is in contrast to homozygous transgenic uPA mice that develop fatal liver insufficiency during the first weeks after birth. The unaffected liver status after Ad.RSV-uPA gene transfer is a substantial advantage of the present invention.

EXAMPLE IIIModification of Urokinase cDNA to Minimize uPA Secretion

To avoid the risk of possible hemorrhage in transduced animals secondary to uPA secretion, this Example describes modifying the gene encoding the uPA protein so that the uPA is retained within the cell.

The methods used to make the modifications of the uPA cDNA to create different ER retention signals are summarized in Fig. 3. A carboxyterminal endoplasmic reticulum retention KDEL amino acid signal was cloned onto the 3' end and referred to as  $\Delta$ C-uPA (Fig. 3B). A  $\Delta$ N-uPA modification was produced by substituting the 25 N-terminal amino acids with an RR-retention signal, together with the transmembrane anchor (TM) separated by a 31 amino acid spacer from the membrane II protein Iip 33 (Fig. 3C), thereby deleting the signal peptide. A third construct contained a combination of the N- and C-terminal modifications ( $\Delta$ N $\Delta$ C-uPA). Recombinant adenovirus vectors that express the unmodified uPA (Example I) or the modified constructs were prepared from expression cassettes that used the RSV-LTR and/or PGK promoters.

The human uPA cDNA (1326bp) cloned as XbaI/Asp718 fragment in pGEM (Promega) was obtained from B.Hinzmann (MDC Berlin) (Nagai et al., Gene, 36:183-188 (1985)). For the modification  $\Delta$ C-uPA, the 3' end of uPA cDNA was extended by a sequence coding for the KDEL signal with additional upstream residues as depicted in Fig. 3. The additional amino acids were included because some of the acidic residues upstream of the tetrapeptide KDEL may be important for the conformation of this domain or for the interaction with the KDEL receptor (Pelham, EMBO J. 7:913-918 (1988)).

The sequence for the modified C-terminus (75nt) was cloned as synthetic oligonucleotide instead of the Bam HI (position:1257)/Asp718 (Fig. 3) fragment of uPA.

The 75nt long ds oligonucleotide was generated by annealing the following phosphorylated ss oligonucleotides (I-V):

I: 5'GATCCCGCAGTCACACCAAGGAAGAGAATGGCCTGGCCCCTC (SEQ ID NO:7)



II: 5'GAAGAAGATACCTCTGAAAAAGATGAGCTCTGAGG (SEQ ID NO:8)

III: 5'TCTTCCTTG GTGTGACTGCG (SEQ ID NO:9)

IV: 5'CAGAGGTATCTTCGAGGGCCAGGCCATTC (SEQ ID NO:10)

V: 5'GTACCCTCAGAGCTCATCTTTTT (SEQ ID NO:11)

5

For the  $\Delta$ N-uPA modification the 25 N-terminal amino acids, including the pre-uPA signal peptide, was substituted by the N-terminal RR-retention signal (Schutze et al., EMBO J., 13:1696-1705 (1994); Strubin et al., Cell 47:619-625 (1986)) together with the transmembrane anchor (TM) separated by a spacer peptide (31 aa) from the membrane II protein Iip33 (Fig. 3C). The Iip33 fragment was cloned by PCR from the Iip33 cDNA, provided by P. Petersen (R.W.Johnson Pharmaceutical Research Institute, San Diego, CA). The forward primer was designed for the Iip33 sequence coding for the Met +1 and following amino acids with a 5' extension containing a XbaI site and a Kozak sequence upstream of the Iip33-ATG:

10

15

20

5'GGCTCTAGATCGCCACC ATG CAC AGG AGG AGA AGC AGG AGC (SEQ ID NO:12)

25

The reverse primer was specific against the Iip33 sequence coding for aa 68-74 with a 5' extension for the in frame fusion with uPA by a Taq I site at position 108 of the uPA cDNA:

5'GTT CGA TGG AAC CTG CTG CTG CTA CAG GAA GTA GGC (SEQ ID NO:13)

30

35

The PCR was carried out from 50 ng Iip33 DNA by Pfu-DNA polymerase (2.5 u) (Stratagene) with 2% DMSO, 300mM dNTP's, 20 uM of each primer and the Pfu reaction buffer #2 (20mM Tris-HCl, pH 8.2, 10mM KCl, 6mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1.5mM MgCl<sub>2</sub>, 0.1% Triton X-100) in 40 cycles (45 sec 95 °C, 45 sec 65 °C, 90 sec 72 °C) followed by 10 min 72°C incubation. The amplification product (251 bp) was separated in a 3% NuSieve agarose, purified and digested with TaqI.

A deletion of the uPA 5' end (XbaI/TaqI (pos:108)) was not possible due to multiple TaqI sites within the uPA gene. Thus, a 526 bp Xba I/ BalI uPA fragment was first

isolated and then recut with TaqI (Fig. 3A). The remaining 421 bp TaqI/BalI fragment was then ligated with the XbaI/TaqI PCR product and the BalI truncated uPA gene/vector. The correctness of the structure of all modifications was confirmed by sequencing from the T7 promoter ( $\Delta$ C-uPA) and Sp6 promoter ( $\Delta$ N-uPA) in pGem.

$\Delta$ N $\Delta$ C-uPA was modified at the N-terminus in the same manner as described for uPA. The resulting modified uPA-genes:  $\Delta$ C-uPA,  $\Delta$ N-uPA, and  $\Delta$ N $\Delta$ C-uPA were cloned as Hind III/Asp718 fragments into the HindIII/Asp718 site of pAdL.1/RSV and pAdL.I/PGK (Fang et al., Gene Therapy 1:247-254 (1994), and Kay et al., Hepatology 21: 815-819 (1995), incorporated herein by reference).

The resulting adenoviral plasmids were designated:

pAd. RSV -  $\Delta$ C-uPA

pAd. RSV -  $\Delta$ N-uPA

pAd. RSV -  $\Delta$ N $\Delta$ C-uPA

pAd. PGK -  $\Delta$ N-uPA

pAd. PGK -  $\Delta$ N $\Delta$ C-uPA

To generate the E1A-recombinant adenoviruses the plasmids were cotransfected with pJM17 into 293 cells by calcium phosphate coprecipitation and plaques were isolated as described in Lieber et al., Hum. Gene Ther. 6: 5-11 (1995), incorporated herein by reference. Virus from positive plaques was expanded, purified by CsCl ultracentrifugation and titered by OD measuring and plaque assay. The supernatant and cell lysate from 10 plaques of each adenovirus were analyzed for uPA in supernatant and cell lysate by ELISA. To generate cell lysate for ELISA analysis an equal number of cells from 12 well plates was pelleted, resuspended in 10mM Tris-Cl pH 7.5, incubated in ice for 10 min, and homogenized in a glass Dounce homogenizer (20 strokes). NaCl was added to a final concentration of 137 mM. The ELISA was as described in Example I above, using monoclonal antibodies against the catalytic and receptor-binding domains of uPA.

To determine how much of the modified uPA protein was localized in the cell or secreted into media, Chinese

Hamster Ovary cells were transduced with enough adenovirus to transduce most of the cells (Examples I and II). Two days later the supernatants and cell lysates were analyzed for uPA antigen by ELISA (Fig. 4A) and enzyme activity by fibrin plaque (Fig. 4B) assays. The procedures for the fibrin plaque assay were modified from those described by Jespersen and Astrup (1983). Fifteen milligrams of fibrinogen were dissolved in 50 mM Tris-HCl, 140 mM NaCl pH7.5 (TBS) and mixed with 2% Agar Noble in TBS, 25mM CaCl<sub>2</sub>, 12.5 mM MgCl<sub>2</sub> at 58-60°C. Five micrograms plasminogen and 60 units thrombin were quickly added to the agar-fibrinogen mix and plated out (thickness 5mm). Fibrin as the product of fibrinogen activation forms polymers which are visible in the agar as cloudy background. Twenty microliter samples or uPA standard dilutions (Sigma) were added in wells with 4 mm diameter and incubated for 4 hr in a humidified chamber. After the diffusion in agar, the uPA forms clear plaques around the cavities. The plaque diameter was measured and the fibrinolytic activity of samples in units/ml was estimated based on a standard curve.

For enzymatic analysis of cell lysates, the cell pellets were resuspended in 0.14M NaCl, 1mM MgCl<sub>2</sub>, 10mM Tris-HCl, pH8.5, 0.5% NP40 and incubated on ice for 20 min.

The results showed that the majority of the unmodified urokinase antigen and enzymatic activity was secreted into the media while all three of the modified uPAs were predominately found in cells. Based on the amount of uPA protein produced from each adenovirus vector, the specific enzymatic activity of the modified uPAs were relatively similar to the wild-type protein. A small proportion of the  $\Delta$ C-uPA and  $\Delta$ N-uPA was found in the media, however, there was virtually no detectable uPA protein in the media of double  $\Delta$ N $\Delta$ C-uPA modified protein. Thus, the double modified construct was used in the subsequent studies. The total amount of uPA production was slightly greater when the RSV-LTR promoter was used compared with the PGK promoter (Fig. 4).

EXAMPLE IVModified uPA Causes Hepatocellular Damage  
Without Secretion into the Bloodstream

This Example demonstrates that Ad.PGK $\Delta$ NAC-uPA can be delivered to mouse hepatocytes *in vivo* and the resulting  $\Delta$ NAC-uPA retained in the hepatocytes, causing hepatocellular damage without secretion of uPA protein into the bloodstream or detectable alterations in hemostasis of the treated animal.

Ad.PGK $\Delta$ NAC-uPA virus was infused into the portal vein of mice and at periodic times as indicated the blood and liver tissue were analyzed for serum uPA (Fig. 5). The results showed that no uPA was detected in the serum of recipient animals over an 11 day period after gene transfer, whereas the protein was detectable at relatively high concentrations in liver tissue on days 3 to 6 after gene transfer. In contrast, in Example I mice infused with a recombinant adenovirus that expressed wild-type uPA had transient serum concentrations of up to 350 ng/ml. In that group, the elevation in the serum urokinase caused a marked increase in the prothrombin time, an indication of hypocoagulability. In this Example, the PTs were found to be in the normal range in animals infused with Ad.PGK- $\Delta$ NAC-uPA (Fig. 5). Furthermore, unlike wild-type uPA, modified uPA expression in hepatocytes did not cause hemorrhage in any of the animals studied. To monitor for hepatocellular injury, serum SGPTs were measured. The elevation in serum SGPT in animals transduced with Ad.PGK- $\Delta$ NAC-uPA was observed between days 3 and 8 after gene transfer (Fig. 5) and indicated that these animals had hepatocellular injury similar to what was previously described with wild-type uPA. Taken together, the modified uPA caused hepatocellular damage without secretion of the protein into the bloodstream or detectable alterations in hemostasis.

As additional evidence that the  $\Delta$ N $\Delta$ C-uPA was localized in the cell, animals were transduced with Ad.RSV-uPA or Ad.PGK- $\Delta$ N $\Delta$ C-uPA and liver sections were immunohistochemically stained for uPA. Although both experimental conditions contained the characteristic histologic appearance of degenerating hepatocytes, the uPA staining patterns showed marked differences. Few of the Ad.RSV-uPA transduced cells contained small amounts of cytoplasmic staining for uPA while a large number of the Ad.PGK $\Delta$ N $\Delta$ C-uPA transduced cells were stained with a pattern suggestive of a membrane localization. Six days after Ad.PGK $\Delta$ N $\Delta$ CuPA adenovirus administration, uPA stained hepatocytes had the characteristic degenerative changes, while the patches of hepatocytes with normal appearance did not stain. This indicates that the normal hepatocytes represent non-transduced regenerating cells.

To determine whether intracellular production of  $\Delta$ N $\Delta$ CuPA caused liver regeneration, animals were infused with either  $5 \times 10^9$  pfu of Ad.RSV-uPA or Ad.PGK $\Delta$ N $\Delta$ C-uPA and were analyzed for incorporation of  $^3$ H-thymidine into hepatic DNA. Both vectors led to similar rates of radioactive incorporation occurring from days 3 to 11 after gene transfer (Fig. 6). Autoradiography showed that the majority of hepatocytes were labelled with radioactivity. Thus, the  $\Delta$ N $\Delta$ CuPA protein produced in hepatocytes caused asynchronous liver regeneration in a manner similar to wild-type uPA.

To establish that the Ad.PGK $\Delta$ N $\Delta$ CuPA and Ad.RSV-uPA adenovirus treated animals were equally susceptible to retrovirus-mediated gene transfer in hepatocytes *in vivo*, 3 and 5 days after adenovirus infusion  $2 \times 10^6$  cfu of LNA1bhAAT retrovirus was infused into the portal vein (Fig. 7). This retrovirus expresses human alpha-1-antitrypsin from transduced cells. The serum hAAT concentrations were similar in both treatment groups, establishing that the new urokinase adenovirus vectors were equally able to allow for retrovirus-mediated hepatic gene transfer.

The results demonstrated herein indicate that both the N- and C- terminal modifications of uPA prevent the

secretion of uPA. The KDEL signal seemed to work better in ER retention as the N-terminal modification. The most effective retention of uPA (no detectable secretion) occurred when both termini were modified as with  $\Delta C\Delta N$ uPA. The pattern of uPA antibody staining in Ad.PGK- $\Delta N\Delta C$ -uPA transduced hepatocytes indicated that the protein is exclusively localized in the ER. The modified uPA caused similar histologic changes found in a uPA-transgenic mouse model (Sandgren et al., Cell 66:245-256 (1991)), in which ultrastructural examination of the livers showed a characteristic cytoplasmic vacuolization. The vesicles were membrane lined, which contained polyribosomes, suggesting an origin from the rough endoplasmic reticulum. The modified uPA of the present invention was present on similar structures. The presence of modified uPA protein on these vacuolized structures was further evidence that the protein is associated with the endoplasmic reticulum membrane.

#### EXAMPLE V

##### tPA Causes Hepatocyte Regeneration Without Hepatocyte Killing

Ad.RSV-tpa is an adenovirus vector that contains the 2.7 kb cDNA encoding human tPA under control of the RSV LTR promoter and the bovine plasminogen activator polyadenylation signal, generated in an E1A-deficient adenovirus after recombination with pJM17, as generally described above in Example 3. The tPA is in a secreted form. Ad.RSV-tpa was administered at a concentration of about  $5 \times 10^9$  pfu to mice via portal vein injection. Proliferation of hepatocytes was measured by <sup>3</sup>H-thymidine incorporation. The Ad.RSV-tpa led to the proliferation of hepatocytes, with peak labeling occurred between days 4 and 5 after adenovirus administration. Three animals per group were analyzed and found to have 90% and 60% hepatocyte labeling on days 4 and 5 respectively. The liver enzymes (SGPT) were in the normal range, and there was no histopathological evidence of hepatocyte injury, in direct contrast to uPA induced liver regeneration. And, whereas

Ad.RSV-uPA results in killing of hepatocytes in culture, the Ad.RSV.tPA had no major effects on hepatocytes in culture. As the tPA did not lead to hepatocyte killing prior to regeneration, the stimulus for regeneration is de novo and not the result of a stimulus that resulted from hepatocyte killing.

#### EXAMPLE VI

This Example describes the construction of an expression unit for a ribozyme library so as to achieve a high level of expression and stability of the expressed ribozyme.

#### Construction and function of the expression cassette

To construct the ribozyme expression vector, oligonucleotides were synthesized in an automatic synthesizer (Applied Biosystems). The genes for vaRNAs I and II were cloned as an XbaI-NsiI fragment (Akusjarvi et al., Proc. Natl. Acad. Sci. USA 77: 2424-2428 (1980) in the XbaI-PstI site of pGEM7zf+ after prior deletion of the AatII site in pGEM. The resulting plasmid was named pGva. Oligonucleotides I 5'CGTCGACTGCTGCAGTGCAGCGTGTGGACCCAACGACACGCGGGCGGTAACCGACGT3' [Seq ID:14] and II 5'CGGTTACCGCCCCGCGTGTGCTTGGGTCCACACGCTGCACTGCAGCAGTCGACGACGT3' [Seq ID:15], (10 ng each), which represent both strands of the sequence to form a loop, were annealed in 20 mM Tris-HCl (pH 7.5)-10 mM MgCl<sub>2</sub> by heating for 5 min. at 85°C and slow cooling to room temperature and cloned into the AatII site within the va gene sequence. The resulting plasmid was designated pGvaL. The 5' end of oligonucleotides III 5'CCGCTCGAG(N)<sub>13</sub>CTGATGAGTCCGTGAGGACGAAA3' [Seq ID:16] and IV 5'TGCATGCAT(N)<sub>11</sub>N<sub>G</sub>TTTCGTCCTCACGGACTCATCAG3' [Seq ID:17], where N<sub>G</sub> is 40% C, 40% G, 10% T, and 10% A, for the randomly mutated ribozymes were phosphorylated with polynucleotide kinase.

Oligonucleotides III and IV (5 µg each) were heated for 5 min. at 85°C in 20 mM Tris-HCl (pH 7.5)-10 mM MgCl<sub>2</sub>

cooled to 65°C, and incubated with 200  $\mu$ M deoxynucleoside triphosphates-(dNTPs) 2.5 U of *Taq* polymerase for 30 min at 65°C. After phenol extraction and ethanol precipitation, the double-stranded oligonucleotides were digested with *Nsi*I-*Xho*I overnight and cloned into the *Sal*I and *Pst*I sites of pGvaL. Ligation products were transformed in highly competent ( $10^{10}$  colonies per  $\mu$ g of DNA) *E. coli* DH5, and plasmid DNA from a pool of  $10^{10}$  different individual clones was prepared. The ribozyme gene library was designated GvaLRz. The corresponding RNAs were designated va, vaL and ValRz.

T7 polymerase-dependent in vitro transcription was performed by incubating 2  $\mu$ g of DNA template, 12.5  $\mu$ l of TKB (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 10 mM 2-mercaptoethanol, 0.1 M KCl, 20% glycerol, 0.5 mM phenylmethanesulfonyl fluoride, 10 mM  $\text{MgCl}_2$ ), 10 U of RNasin, 2.5  $\mu$ l of 5 mM NTPs (or 5 mM ATP, GTP, and TTP plus 20  $\mu$ Ci of [ $^{32}$ P]CTP and 1 mM CTP), 5 mM  $\text{MgCl}_2$ , 2.5  $\mu$ l of 10 mM dithiothreitol, 100 U of T7 RNA polymerase (Biolabs) in a total volume of 25  $\mu$ l at 37°C for 60 min. After digestion with 23 U of DNase I, the transcripts were purified twice by phenol extraction and ethanol precipitated. The amount of RNA synthesized was estimated after gel electrophoresis in an ethidium bromide-stained agarose gel calibrated with concentration markers. In a standard reaction, 5 to 8  $\mu$ g of RNA per 25- $\mu$ l reaction volume was synthesized.

#### Function and stability of the chimeric ribozyme RNA.

Cleavage reactions with ribozymes were performed in vitro. For analytical analysis, 100 nM ribozyme and 100 nM in vitro-transcribed substrate human growth hormone (hGH) RNA or HCV plus RNA (type 1 $\alpha$ ) were mixed in a 15- $\mu$ l reaction volume containing 50 mM Tris (pH 7.5) and 1 mM EDTA. For heat denaturation, the mixture was boiled at 95°C for 90 s and quickly cooled on ice.  $\text{MgCl}_2$  (10 mM) was added, and the mixture was incubated at 37°C for 30 or 60 min. The reaction was stopped by addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), the mixture was heated at 95°C for 2 min., and



the products were analyzed in a 4 or 6% polyacrylamide-8 M urea gel in Tris-borate EDTA buffer.

Total-cell RNA was extracted by the guanidinium-phenol method (Chomczynski and Sacchi, Anal. Biochem. 162:156-159 (1987)) or with the RNaid Plus Kit (Bio 101, La Jolla, CA). Total hGH-RNA was extracted from a hGH expressing cell line. Total HCV (type 1b)-RNA was extracted from HCV positive human liver.

For northern blot analysis, RNA samples were fractionated on a 1.5% agarose gel containing 2.2 M formamide and transfected to Hybond N+ nylon membrane. Northern (RNA) hybridization was carried out with <sup>32</sup>P-labelled probes by the method described by Westneat et al., Nucl. Acids. Res. 16:4161-4170 (1988)). HCV and vaDNA sequences were labelled by standard random-priming techniques with DNA pol 1 (Klenow fragment).

#### Creation of a functional ribozyme library.

**Cleavage of cellular RNA in vitro by ribozymes from the library.** Purified total-cell RNA was used as the source of mRNA.

Cleavage was carried out at physiological pH (50 mM Tris-HCl [pH 7.5]) at 37°C for 1 h in a 15-μl reaction volume with or without prior heat denaturation (for 90 s at 95°C). The cleavage products were analyzed in a 2% ethidium bromide-stained NuSieve agarose gel and could be detected as a smear between the 18S and 28S rRNAs and below the 18S rRNA. In some cases, the 5'-OH groups of cleavage products were phosphorylated with [<sup>32</sup>P]ATP by using polynucleotide kinase and quantified on a Fuji Phosphorimager after polyacrylamide gel electrophoresis.

For the cleavage reaction purified total RNA (1 μg per reaction) and 10 μg of vaL (as control) or vaLRz (library) RNA, which were synthesized by T7 polymerase, were mixed and incubated in 50 mM Tris-HCl-10 mM MgCl<sub>2</sub> for 1 h at 37°C with or without prior heat denaturation. No significant self-digestion of ribozymes was observed. The ribozyme cleavage was three times more efficient when the reaction mixture was

heat denatured. Without addition of 10 mM  $MgCl_2$ , no specific cleavage reaction was detected. For further analyses, cleavage products were used without prior heat denaturation.

After the reaction, RNA was purified with oligo(dT)-cellulose (PolyAtract mRNA Isolation System; Promega) according to the manufacturer's specifications. After purification, 0.05 to 0.5  $\mu g$  of RNA was annealed with 2.5  $\mu M$  oligo(dT) primer (Promega) for 10 min. at 70°C, and unbound primer was separated by centrifugation through a 30,000-molecular-weight-cutoff filter unit (Millipore). Reverse transcription was performed with 200 U of superscript II reverse transcriptase (Bethesda Research Laboratories) at 37°C for 1 h. To eliminate free primers, cDNA-RNA hybrids were purified with Gene Clean (Bio 101). The cDNA-RNA hybrid in 30  $\mu l$  of  $H_2O$  was boiled for 2 min. and cooled on ice. Tailing was carried out for 15 min. 37°C in a total volume of 50  $\mu l$  containing 200  $\mu M$  dGTP, 20 U of terminal deoxynucleotidyltransferase (Bethesda Research Laboratories), and tailing buffer.

A 5% portion of the tailing-reaction mixture was used for the first PCR with 200  $\mu M$  dNTP, 1.5 mM  $MgCl_2$ , and 2.5 U of Taq polymerase in buffer containing 2% dimethyl sulfoxide, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), and 0.1% Triton X-100 in a 100- $\mu l$  reaction volume. The initial seven cycles (30 s at 95°C, 30 s at 42°C, and 90 s at 72°C) were run in the presence of 15  $\mu M$  C-Primer (5'GAGAATTCTAGAGGATCCCCCCCCCCCC3' [Seq ID:18]) only. After addition of 250  $\mu M$  hGH-specific primer (5'GAGAATTCCAAGGCCAGGAGAGGCACTGGGGA3' [Seq ID:19]), which is specific for a region immediately upstream of the poly(A) signal of the genomic hGH gene. PCR-primers for selecting HCV ribozymes are directed against sequences in the 5' untranslated region and core region of the HCV genome (see Fig. 9), and comprised: C1+: 5'GTAAACTCCACCAACGATCT [Seq ID:20]; C2+: 5'GAAGATAGAGAAAGAGCA [Seq ID:21]; C3+: 5'ACCCCATGAGGTCGGCGAA [Seq ID:22]; C1-: 5'CTGTGAGGAAGTACTGT [Seq ID:23]; and C2-: 5'CACGCAGAAAGCGTCTAGCC [Seq ID:24]. PCR was done for 40 cycles (60 s at 94°C and 90 s at 72°C or 60 s at 95°C, 45 s at

65°C, and 60 s at 72°C). The reaction mixture was run on a native 5% PAA gel in Tris-acetate buffer. HCV specific bands were identified by Southern blot with a 32P-labelled 730bp fragment (XbaI/ClaI) of pTET/HCV5'T7G3'AFL (Lemm, Kolyakhov, Heise, Feinstone and Rice). Specific bands were cut out and purified with Gene Clean. The resulting fragments were cloned in the pGEM-T II vector system (Promega). Clones corresponding to the 5' end of individual RNA downstream cleavage products were sequenced with the fmole sequencing kit (Promega).

#### Identification of specific ribozymes in the library.

**Amplification of ribozymes.** Ribozyme genes from the library were amplified as follows. PCR was performed by incubating 50 ng of plasmids from the ribozyme library, 20 µM each upstream and downstream primer (which are specific for the sequences around the GTC/CTC site and 2.5 U of Taq polymerase in buffer containing 2% dimethyl sulfoxide, 50 mM KCl, 10 mM Tris-HCl (pH 7.9), and 0.1% Triton X-100 in a 100-µl reaction volume for 40 cycles (45 s 95°C, 45 s at 52°C, and 45 s at 72°C). Specific fragments were prepared as described above, digested with XhoI-NsiI, cloned into the SalI and PstI sites of GvaL, and sequenced with the T7 promoter primer.

**Cell culture.** Cells were grown in Dulbecco's modified Eagle's medium containing 200 mM asparagine, 200 mM proline, 200 mM glutamine, and 10% fetal calf serum (GIBCO, Grand Island, NY) under a 5% CO<sub>2</sub> atmosphere.

**Transfection.** Plasmids were purified by two rounds of cesium chloride gradient centrifugation. For transfection of CHO cells, a modification of the standard calcium phosphate coprecipitation method was used (Lieber et al., Nucleic Acids Res. 17:8485-8493 (1989)). DNA (10µg) in 220 µl of H<sub>2</sub>O was mixed with 30 µl of 2 M CaCl<sub>2</sub> and 250 µl of 2x HBS (50 mM HEPES, 280 mM NaCl, 1.5 mM sodium phosphate [equal amounts of mono- and dibasic] [pH 6.96] was added dropwise while the mixture was vortexed. The precipitate was added to 5 mL of culture medium in 25-cm<sup>2</sup> tissue culture flasks containing 2.5 x 10<sup>5</sup> CHO cells that had been seeded the day before. Cells

were transfected with a mixture of 8  $\mu$ g of pTET HCV (Lemm et al., supra) test plasmid, and 1  $\mu$ g of pSV2neo (Southern and Berg, J. Mol. Appl. Genet. 1: 327-341 (1982)). After 48h, 1/10 of the cells were subjected to selection with 600  $\mu$ g of G418 per ml to generate pools of about 100 colonies in order to test for stable expression levels. The cell line was named HCV-CHO.

Six ribozymes specific for HCV types 1a and 1b RNA were identified using the above described protocol. Fig. 9 shows cleavage sites for the HCV ribozymes, Rz1-Rz6, on the HCV RNA plus and minus strands. The HCV ribozyme target sequences are as follows, based on a cDNA sequence that corresponds to the HCV type 1a and type 1b RNA, where the putative ribozyme cleavage sites are indicated by a "-":

ribozyme 1 (Rz1): CGGGAGGTCTCGTA [Seq ID:1] (5' UTR, nucleotides 317 to 332; plus strand), Rz2: GCACCATGAGCAGAA [Seq ID:2] (nucleotide 337 to 352; minus strand), Rz5: CTAAACCTCAAAGAAA [Seq ID:3] (core, nucleotide 355 to 370; plus strand), Rz4: CCAACCGTCGCCACAG [Seq ID:4] (core, nucleotide 385 to 401; plus strand), Rz3: CCCACAGGACGTCAAG [Seq ID:5] (core, nucleotide 385 to 410; minus strand), and Rz6: GGTAAGGTCATCGATA [Seq ID:6] (core, nucleotide 699 to 714; plus strand).

## EXAMPLE VII

### Ribozyme-Mediated Inhibition of HCV RNA

Sequences encoding the HCV-specific ribozymes were cloned into the pXCJL.1 plasmid (Spessot et al., Virology 168:378-387 (1989)) to produce recombinant adenovirus vectors (Fig. 8). The effect of adenovirus-mediated gene transfer of the HCV ribozymes on HCV RNA in an HCV-RNA expressing cell line was then determined.  $1 \times 10^6$  CHO cells that express about 200 copies of plus strand HCV RNA per cell were incubated for 4 hrs. with 1000 pfu/cell of recombinant adenovirus vectors (the amount of virus that transduces 100% of CHO cells) that express ribozymes 1 through 6 or an

irrelevant control vector. Forty hours later, total nucleic acid was extracted from harvested cells and HCV specific RNA was quantified by solution hybridization with an oligonucleotide specific for HCV type 1a (nt 324-352) within the 5' NCR. The results are shown in Fig. 10, where the values represent the mean and standard deviations for five different experiments. A minor non-specific HCV signal was seen in normal CHO cells, and transduction with an irrelevant control resulted in a minor reduction in HCV RNA signal. Ribozymes 2 and 3 are directed against the negative HCV RNA strand and had no specific effect on plus strand HCV RNA concentrations.

#### EXAMPLE VIII

##### Ribozyme-Mediated Inhibition of hGH in Transgenic Animals

To confirm that sequence specific ribozymes can be targeted to the liver for expression in animals, transgenic mice were created that expressed human growth hormone (hGH) and then treated with adenovirus vectors capable of expressing hGH-specific ribozymes. Fig. 11A shows the concentration of hGH serum concentrations in hGH-transgenic mice after ribozyme therapy, where hGH levels were estimated by enzyme-linked immunosorbent assay (ELISA) as described in Lieber et al., Meth. Enzymol. 217: 47-66 (1993)). Serum samples were analyzed for growth hormone at varying times after recombinant adenovirus administration. Fig. 11B shows hepatic mRNA quantitation in transgenic mice after ribozyme therapy, where hepatic mRNA levels were determined by solution hybridization (Townes et al., 1985; Durnam and Palmiter, 1983). Ad/RSVhAAT was used as a control vector, Ad.polIII.Rz is adenovirus containing the hGH ribozyme driven by the adenovirus val promoter, and Ad.T7Rz+Ad.T7 pol is a 1:1 mixture of the adenovirus expressing nuclear T7 polymerase from the PGK promoter and an adenovirus containing the T7 promoter used to drive transcription of the hGH ribozyme, (Lieber et al., Meth. Enzymol. 217:47-66 (1993)). The effective inhibition of hGH

by the adenovirus encoding the hGH-specific ribozyme indicates that HCV-specific ribozymes can also be effective in vivo in inhibiting HCV RNA.

#### EXAMPLE IX

##### Ribozyme-Mediated Inhibition of HCV-RNA Expression in HCV-Infected Human Hepatocytes

This Example confirms that sequence specific ribozymes are functional against naturally synthesized HCV RNA molecules within infected hepatocytes.

Human hepatocytes were isolated from a patient with HCV (type 1b) infection undergoing liver transplantation. Approximately  $1 \times 10^6$  viable (trypan blue exclusion) hepatocytes were plated into  $25\text{cm}^2$  flasks coated with collagen I. After 6 hours many of the hepatocytes had not plated. The number of plated hepatocytes (determined by counting) was about  $1 \times 10^5$  per flask. The hepatocytes were infected with various recombinant adenovirus vectors (Ad.contr. = adenovirus vector control; Ad.Rz1-6 = adenovirus containing single expression cassettes for ribozymes 1-6 mixed in equal proportions; and Ad.Rz1-6multim.= adenoviruses containing three multimers of the ribozyme expression cassettes mixed in equal proportions) at an MOI of 100. Forty-eight hours later the cells were isolated and total RNA was extracted. One microgram of RNA from each sample was subjected to HCV quantitation by solution hybridization in a manner described above (Example VII). Specifically, oligonucleotide probe from sequences located near the AUG start site (nt409-TTG ACG TCC TGT GGG CGA CGG TTG GTG-nt383) was used. A standard HCV RNA curve was determined using *in vitro* transcribed HCV RNA.

The results, shown in Fig. 12, indicate there was an average of 110 pg HCV RNA per  $10^5$  cells in the isolated hepatocytes. After incubation with adenoviruses expressing the HCV ribozymes, there was no detectable HCV RNA (signal equal to non-infected human hepatocytes). The effect was specific because HCV infected hepatocytes which received a

control adenovirus contained similar amounts of HCV RNA as non-adenovirus treated hepatocytes. The experiments represent the mean with standard deviations (n=four samples per experimental condition). Normal hepatocytes contained a small amount of HCV negative cross reacting signal.

These results thus confirm the findings obtained in the CHO cells that synthesize a full length HCV RNA but not virus. Importantly, this provides direct evidence that the ribozymes are functional against HCV RNA that occurs naturally within HCV infected hepatocytes.

All publications, patents and foreign patent publications are herein incorporated by reference to the same extent as if each individual publication, patent or patent publication was specifically and individually indicated to be incorporated herein by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent that certain changes and modifications may be practiced within the scope of the appended claims.

WHAT IS CLAIMED IS:

- 1                   1. A method for producing a gene product of  
2 interest in the liver of a patient, comprising:  
3                   transducing native hepatocytes which have been  
4 removed from a patient with a vector which encodes the gene  
5 product of interest;  
6                   transducing *in situ* native hepatocytes of the  
7 patient with an adenoviral vector which encodes tPA or uPA;  
8 and  
9                   returning the transduced hepatocytes to the patient  
10 and providing conditions conducive to the expression of the  
11 gene product of interest.
- 1                   2. The method of claim 1, wherein the tPA or uPA is  
2 not secreted by the adenoviral-transduced hepatocytes.
- 1                   3. The method of claim 1, wherein the tPA is  
2 secreted by the adenoviral-transduced hepatocytes.
- 1                   4. The method of claim 1, wherein the gene product  
2 of interest is a blood coagulation protein.
- 1                   5. The method of claim 1, wherein the vector which  
2 encodes the gene product of interest is a retroviral vector.
- 1                   6. The method of claim 1, wherein the transduced  
2 hepatocytes are returned to the patient by infusion through  
3 the spleen or portal vasculature.
- 1                   7. The method of claim 1, wherein the native  
2 hepatocytes of the patient are transduced with the adenoviral  
3 vector at least 1 to 2 days before the transduced hepatocytes  
4 are returned to the patient.
- 1                   8. A method for expressing a gene product of  
2 interest in hepatocytes of the liver of an individual in need  
3 of said gene product, comprising:



4 transducing hepatocytes of the individual *in situ*  
5 with an adenoviral vector which encodes tPA; and  
6 transducing hepatocytes of the individual with a  
7 vector which encodes the gene product of interest,  
8 whereby the gene product of interest is expressed by  
9 the hepatocytes of said individual.

1 9. The method of claim 8, wherein the hepatocytes  
2 are transduced *ex vivo* with the vector which encodes the gene  
3 product of interest and are returned to the individual.

1 10. The method of claim 8, wherein the adenoviral  
2 vector encodes human tPA.

1 11. The method of claim 9, wherein the tPA is  
2 secreted by the adenoviral-transduced hepatocytes.

1 12. The method of claim 9, wherein the vector which  
2 encodes the gene product of interest is a retroviral vector.

1 13. The method of claim 9, wherein the hepatocytes  
2 of the patient are transduced with the adenoviral vector at  
3 least 1 to 2 days before the hepatocytes are transduced with  
4 the vector encoding the gene product of interest.

1 14. The method of claim 13, wherein the hepatocytes  
2 are transduced with the adenoviral vector about 4 days before  
3 the hepatocytes are transduced with the vector encoding the  
4 gene product of interest.

1 15. A method for inhibiting hepatitis C virus RNA  
2 in cells, comprising:  
3 transducing said cells with a recombinant adenovirus  
4 which encodes a ribozyme specific for hepatitis C virus RNA,  
5 and maintaining the transduced cells under conditions whereby  
6 the ribozyme is expressed and inhibits the hepatitis C virus  
7 RNA.

1           16. The method of claim 15, wherein the cells are  
2 hepatocytes.

1           17. The method of claim 16, wherein the hepatocytes  
2 are a hepatoma cell line.

1           18. The method of claim 16, wherein the hepatocytes  
2 are from an HCV-infected individual.

1           19. The method of claim 16, wherein the infected  
2 hepatocytes are in an individual infected with hepatitis C  
3 virus.

1           20. The method of claim 19, wherein the transducing  
2 step comprises administering said recombinant adenovirus to  
3 the individual by infusion.

1           21. The method of claim 20, wherein the infusion is  
2 via the portal vein or bile duct.

1           22. The method of claim 15, wherein the ribozyme is  
2 a hammerhead ribozyme.

1           23. The method of claim 15, wherein the ribozyme  
2 specifically cleaves hepatitis C RNA in a HCV 5'non-coding  
3 sequence, core sequence, or NS-5 sequence.

1           24. The method of claim 23, wherein the ribozyme is  
2 selected from a ribozyme specific for an HCV RNA transcript  
3 corresponding to a cDNA sequence comprising CGGGAGGTCTCGTA  
4 [Seq ID:1] (nucleotide 317 to 332), GCACCATGAGCACGAA [Seq  
5 ID:2] (nucleotide 337 to 352), CTAAACCTCAAAGAAA [Seq ID:3]  
6 (nucleotide 355 to 370), CCAACCGTCGCCCACAG [Seq ID:4]  
7 (nucleotide 385 to 401), CCCACAGGACGTCAAG [Seq ID:5]  
8 (nucleotide 385 to 410), or GGTAAGGTCATCGATA [Seq ID:6]  
9 (nucleotide 699 to 714).

1           25. The method of claim 15, wherein the adenoviral  
2 vector encodes at least two ribozymes specific for hepatitis C  
3 virus mRNA.

1           26. The method of claim 15, wherein the recombinant  
2 adenovirus is administered to the hepatocytes in an amount of  
3 from 10 to 100 adenovirus particles per hepatocyte.

1           27. The method of claim 15, wherein the recombinant  
2 adenovirus is administered to the hepatocytes in an amount  
3 sufficient to eradicate hepatitis C virus from the  
4 hepatocytes.

1           28. The method of claim 15, wherein the recombinant  
2 adenovirus further comprises a ligand specific for a receptor  
3 on said hepatocytes.

1           29. A composition for inhibiting hepatitis C virus  
2 RNA in a cell, which comprises a recombinant adenovirus  
3 encoding at least one ribozyme specific for hepatitis C virus  
4 RNA.

1           30. The composition of claim 29, wherein the  
2 recombinant adenovirus encodes two different ribozymes  
3 specific for hepatitis C virus RNA.

1           31. The composition of claim 29, wherein the  
2 ribozyme is a hammerhead ribozyme.

1           32. The composition of claim 29, wherein the  
2 ribozyme is specific for a HCV RNA transcript corresponding to  
3 a cDNA sequence comprising CGGGAGGTCTCGTA [Seq ID:1]  
4 (nucleotide 317 to 332), GCACCATGAGCACGAA [Seq ID:2]  
5 (nucleotide 337 to 352), CTAAACCTCAAAGAAA [Seq ID:3]  
6 (nucleotide 355 to 370), CCAACCGTCGCCCACAG [Seq ID:4]  
7 (nucleotide 385 to 401), CCCACAGGACGTCAAG [Seq ID:5]  
8 (nucleotide 385 to 410), or GGTAAGGTCATCGATA [Seq ID:6]  
9 (nucleotide 699 to 714).

1                   33. A composition which comprises a ribozyme  
2 specific for an HCV RNA transcript corresponding to a cDNA  
3 sequence comprising CGGGAGGTCTCGTA [Seq ID:1] (nucleotide 317  
4 to 332), GCACCATGAGCACGAA [Seq ID:2] (nucleotide 337 to 352),  
5 CTAAACCTCAAAGAAA [Seq ID:3] (nucleotide 355 to 370),  
6 CCAACCGTCGCCCACAG [Seq ID:4] (nucleotide 385 to 401),  
7 CCCACAGGACGTCAAG [Seq ID:5] (nucleotide 385 to 410), or  
8 GGTAAGGTCATCGATA [Seq ID:6] (nucleotide 699 to 714).

1/12

FIG. 1A

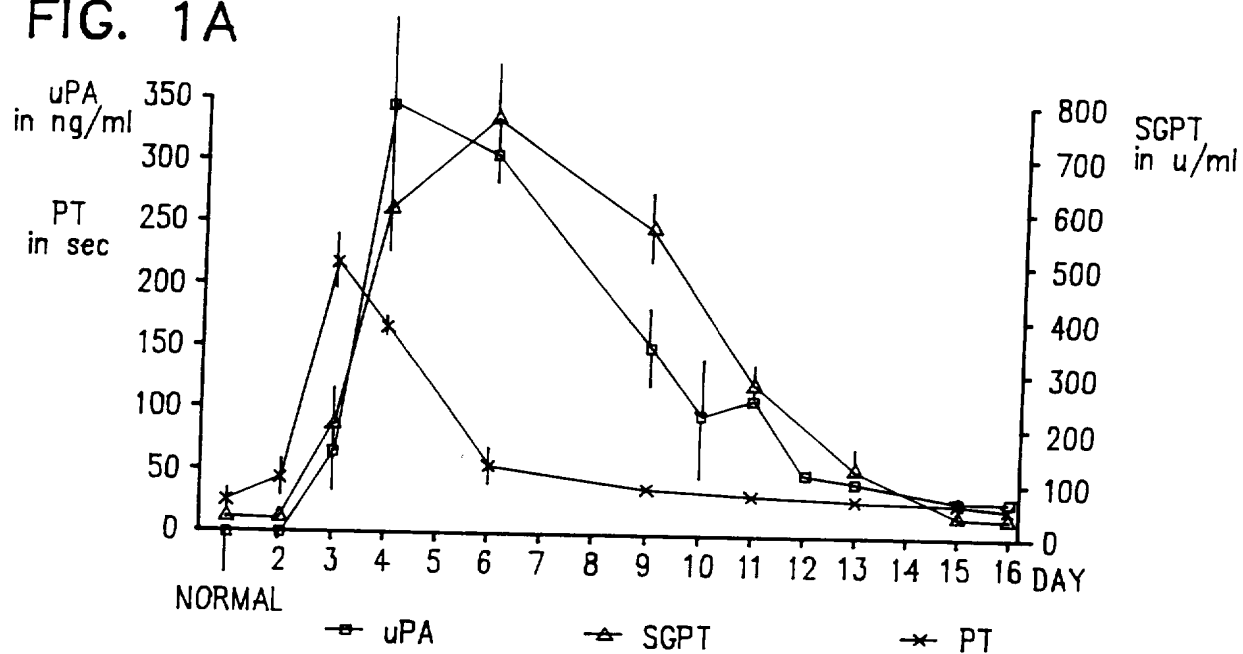
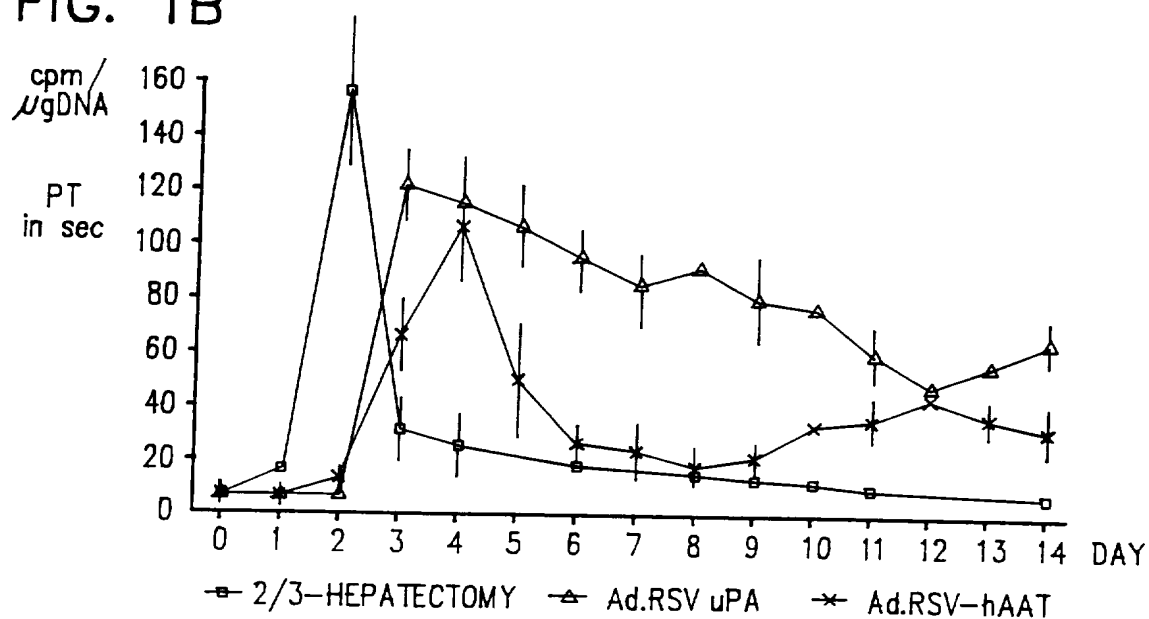


FIG. 1B



2/12

FIG. 2A

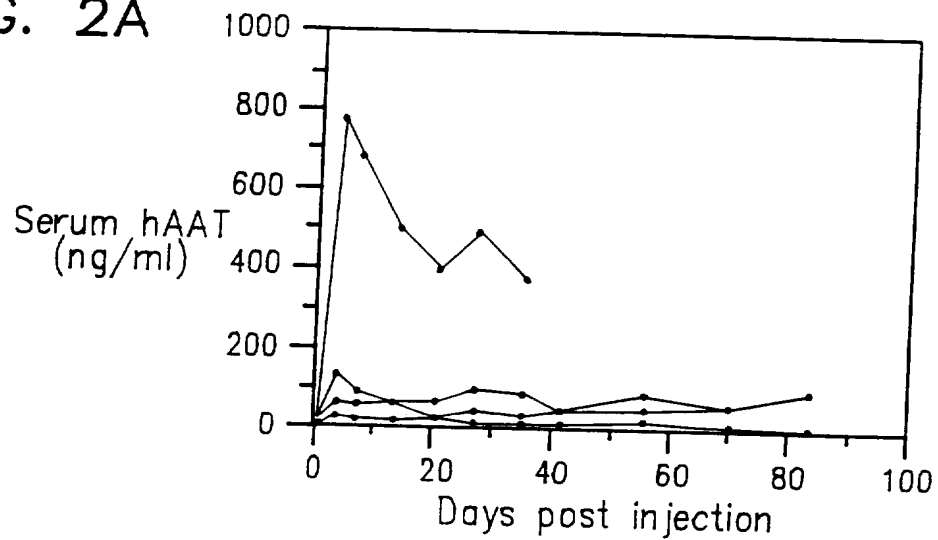


FIG. 2B

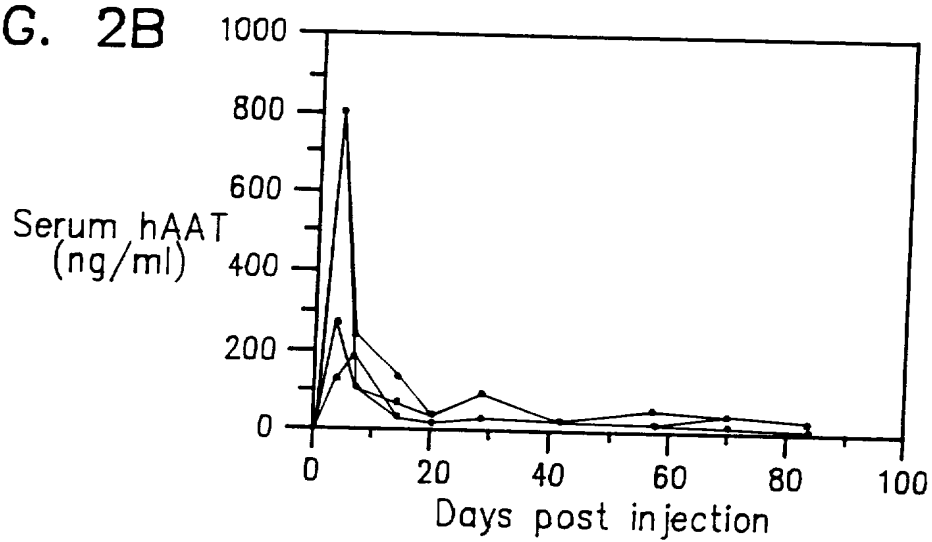
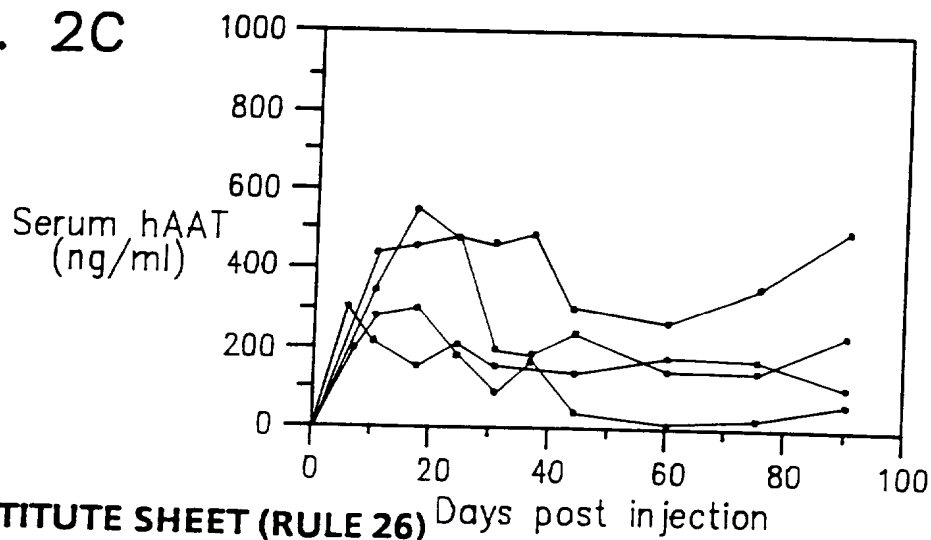


FIG. 2C



3/12

FIG. 2D

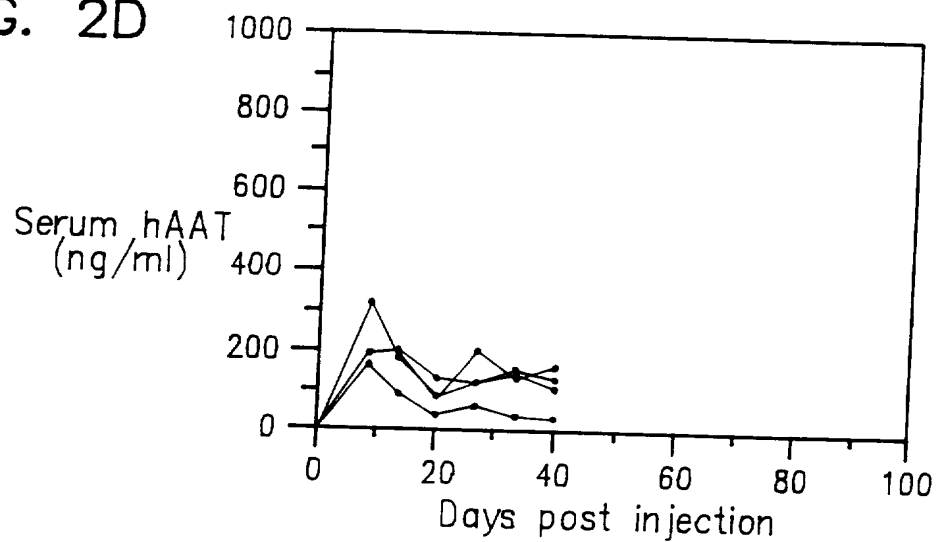


FIG. 2E

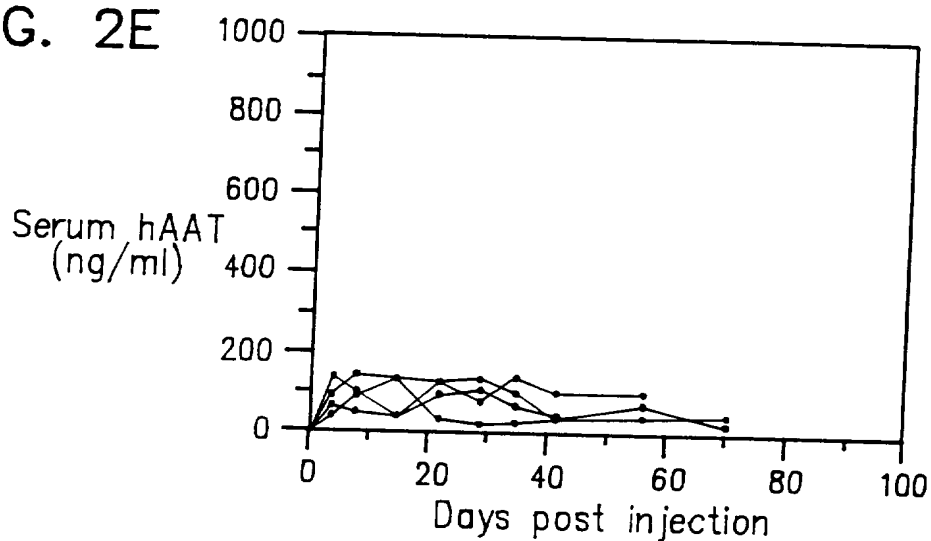
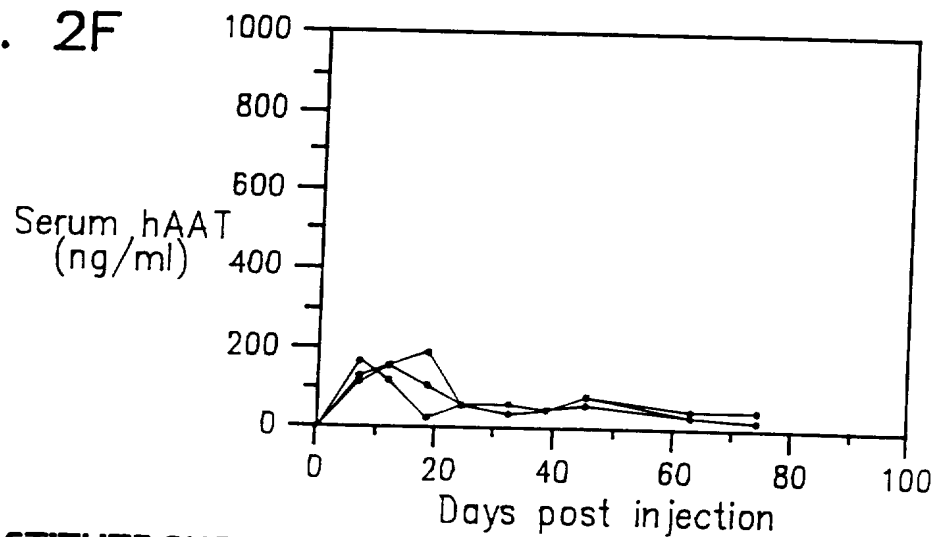
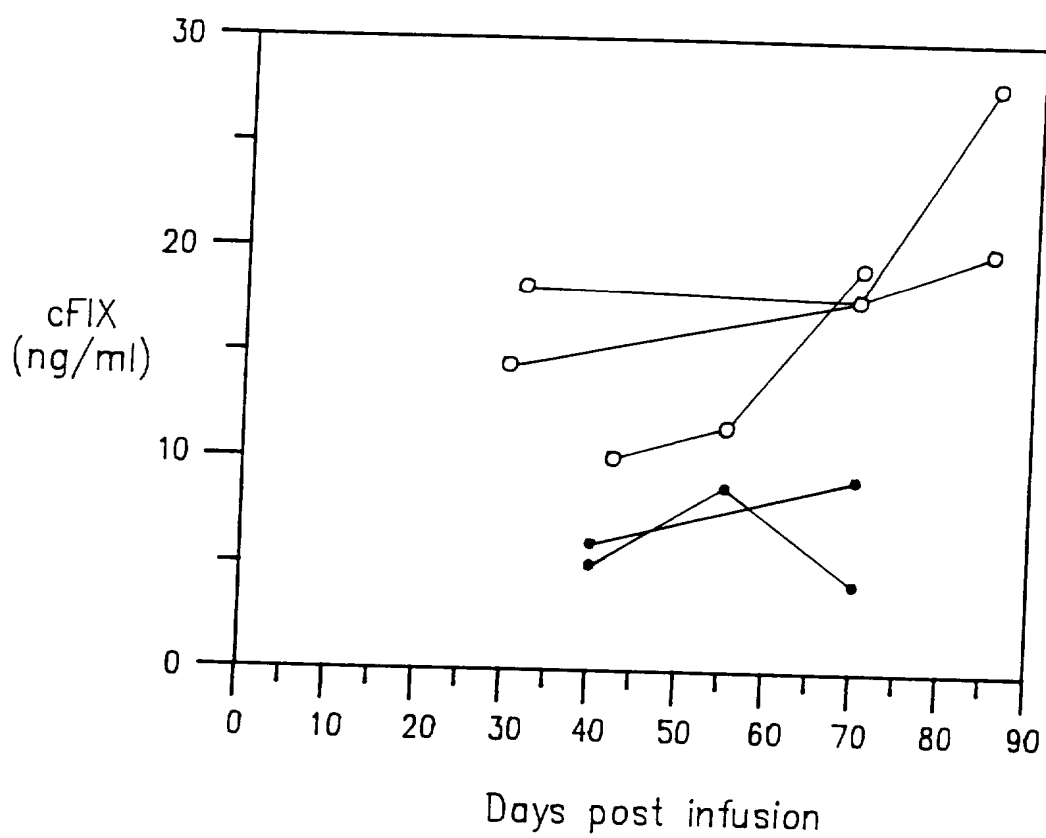


FIG. 2F



4/12

FIG. 2G





5/12

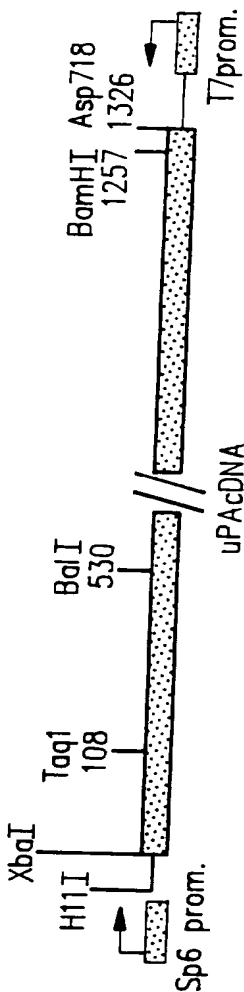


FIG. 3A

FIG. 3B

C-terminal KDEL-signal(synthetic)

uPA .... WIRSHTKEEENGLALCT C-Term

ΔC-uPA .... WIRSHTKËENGLALËËDTSEKDELCT C-Term

..TCGATCCGC...  
BamHI

..GAGCTCTGAGGGTACC  
Asp718

N-terminal lip33-signal(PCR cloning)

pre-uPA

signal peptide

(N-Term)

MRALLARLLLCVLVSDSKG

31

pre-uPA

(N-Term)

RR

15

TM

25

YQQQVPSN.....

...CCATCGAAC...

TaqI

GGCTCTAGATCGCCACC [ATG] CAC ...

XbaI Kozak

FIG. 3C

6/12

FIG. 4A

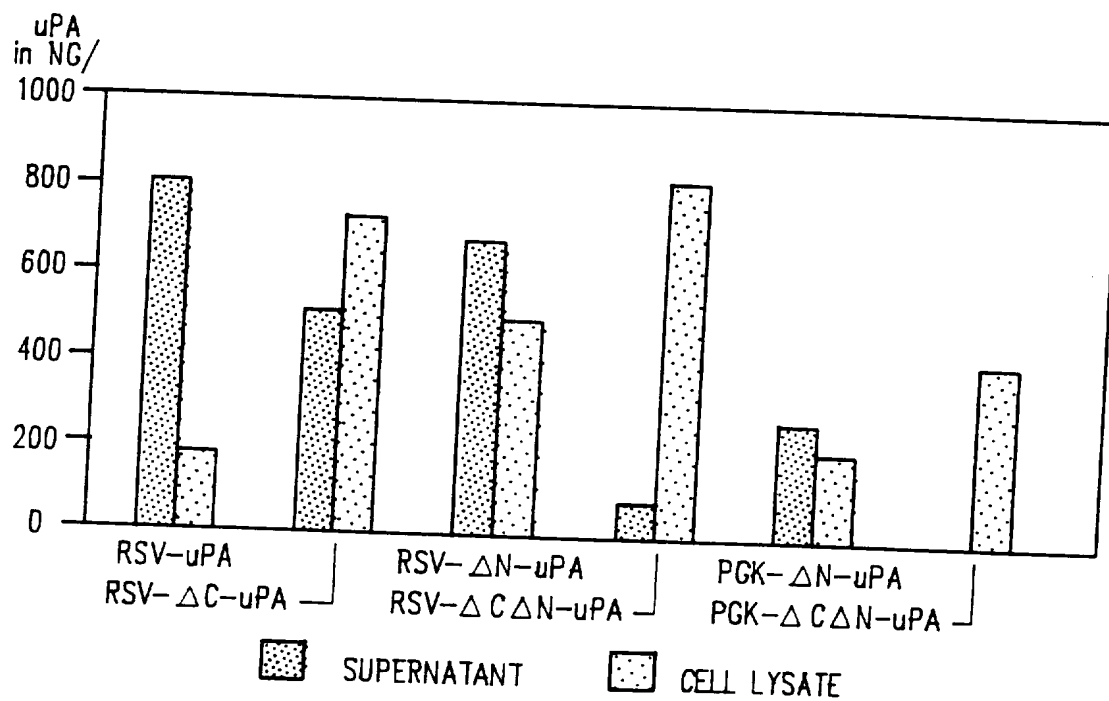
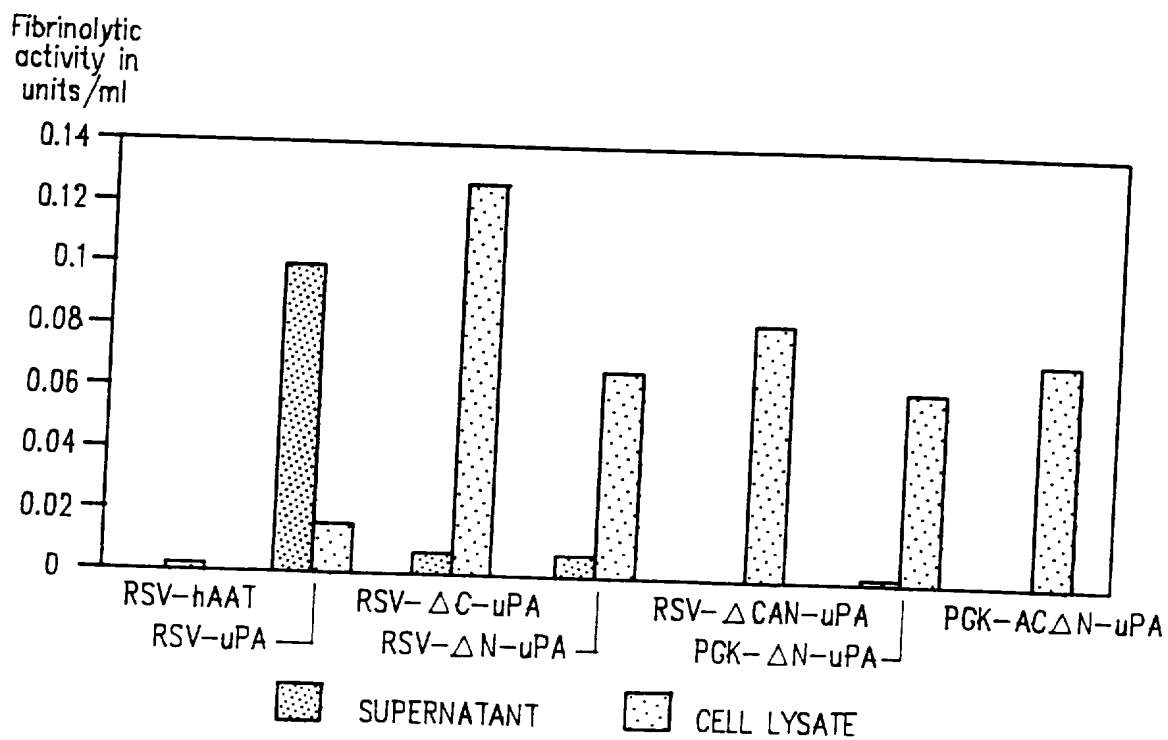


FIG. 4B



7/12

FIG. 5

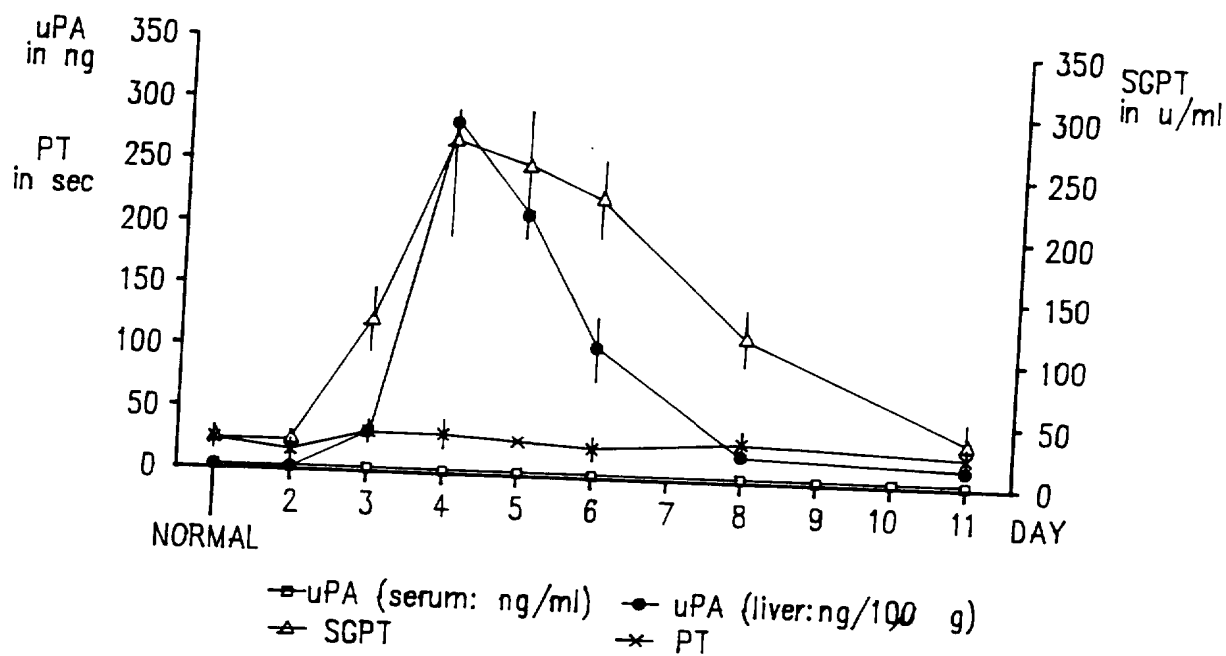
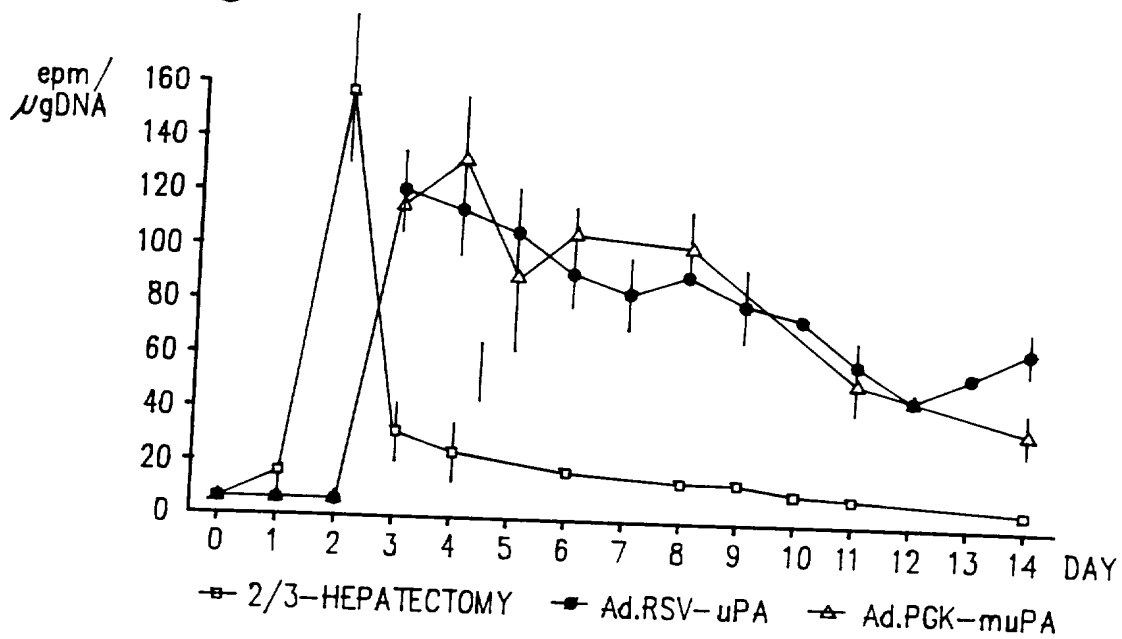


FIG. 6



8/12

FIG. 7A

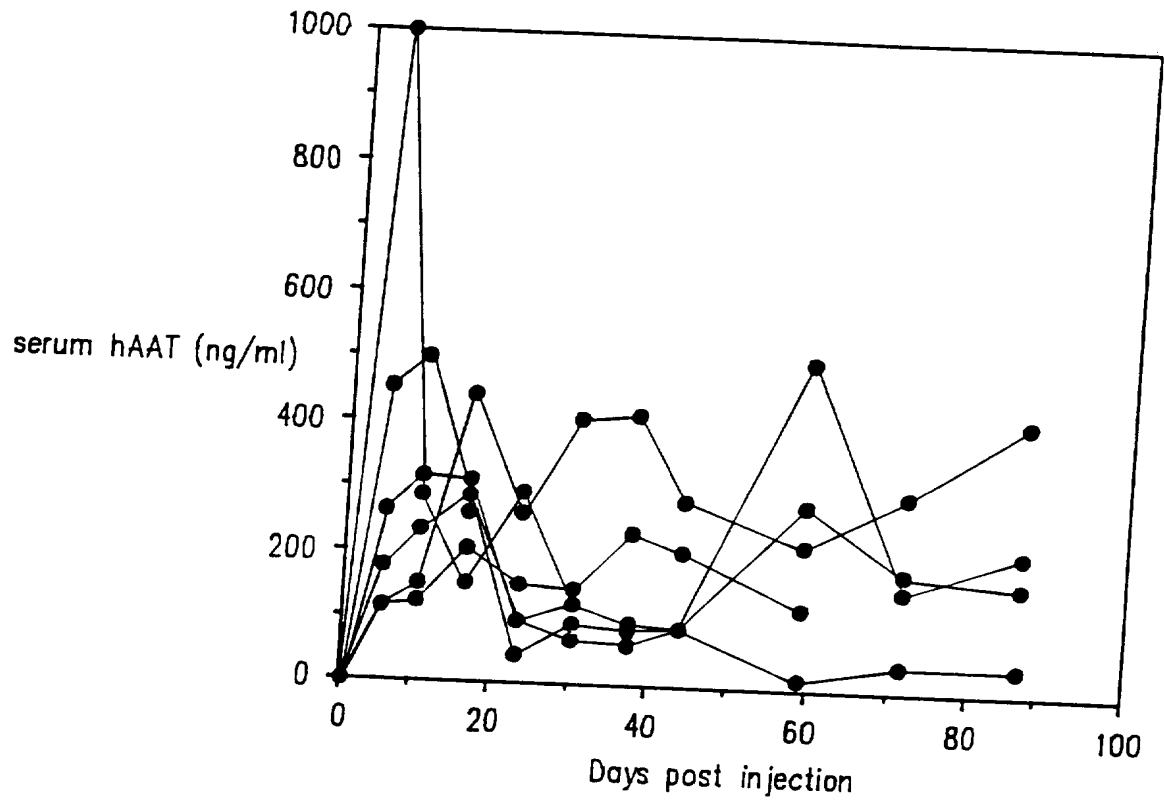
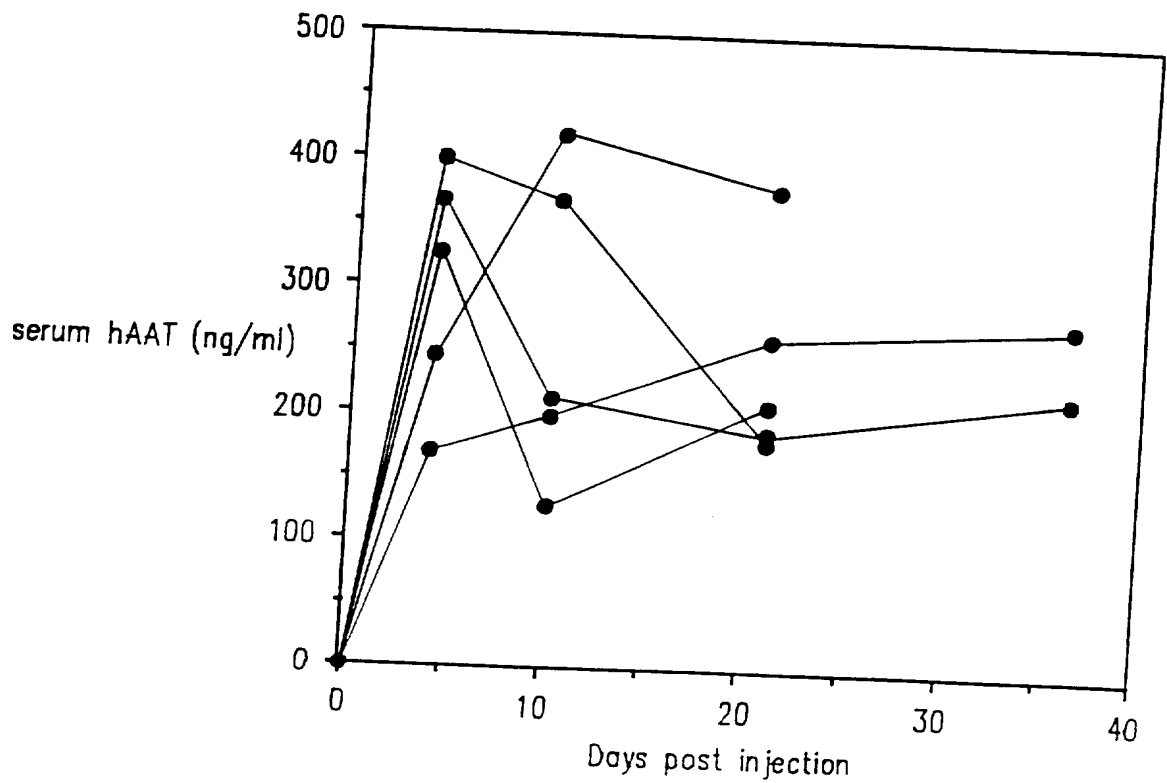
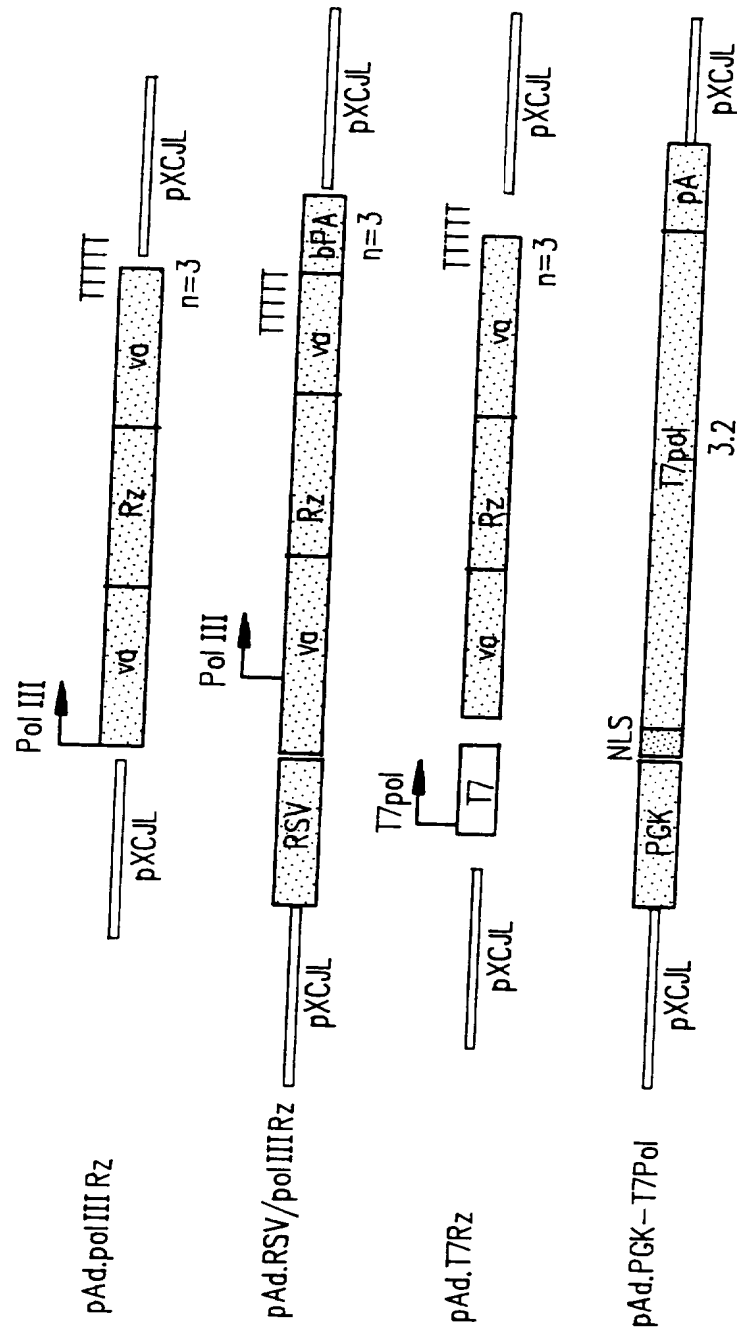


FIG. 7B



9/12

FIG. 8



10/12

FIG. 9

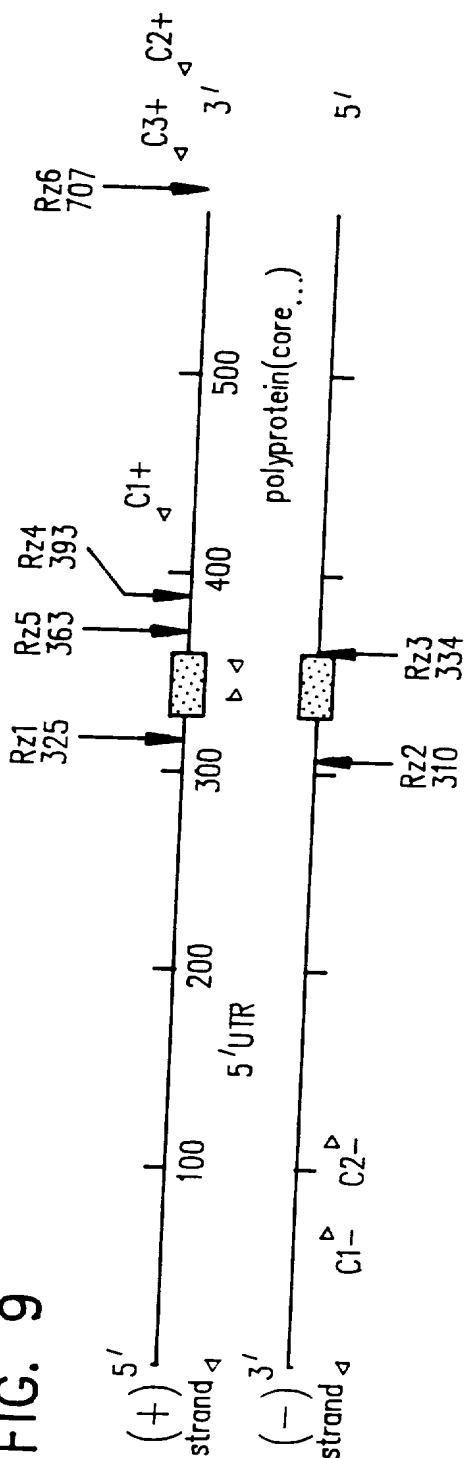
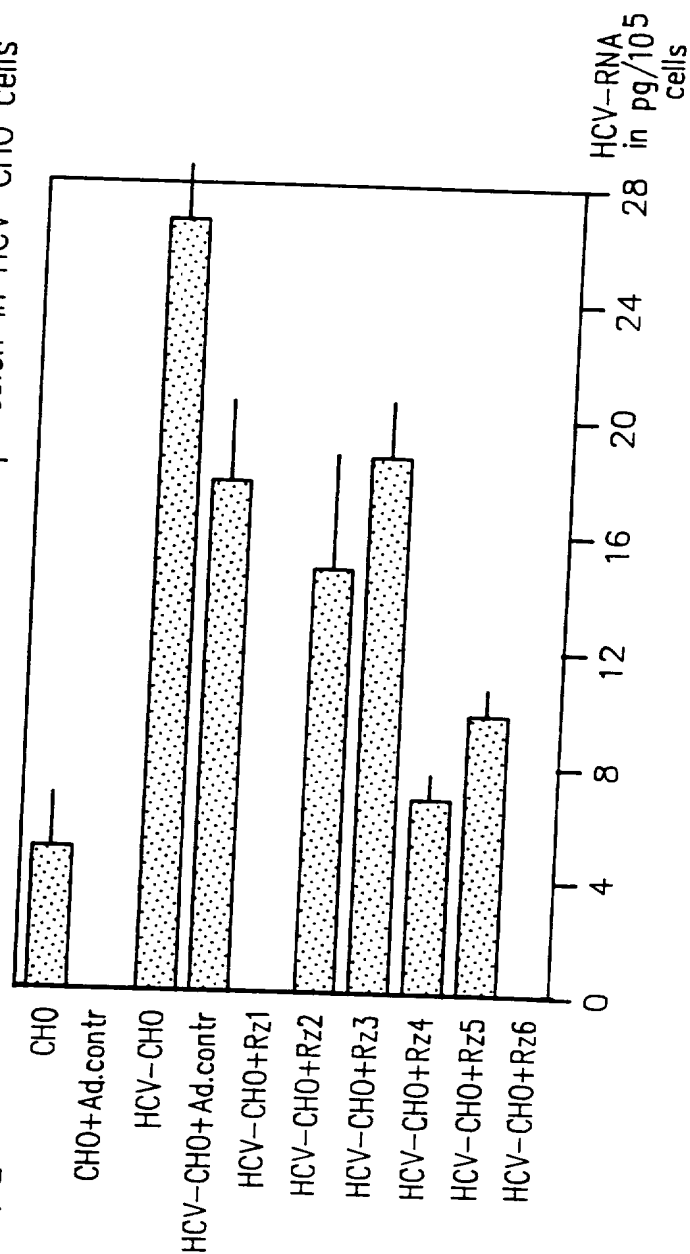


FIG. 10 Effect of HCV-ribozyme on HCV-RNA expression in HCV-CHO cells



11/12

FIG. 11A

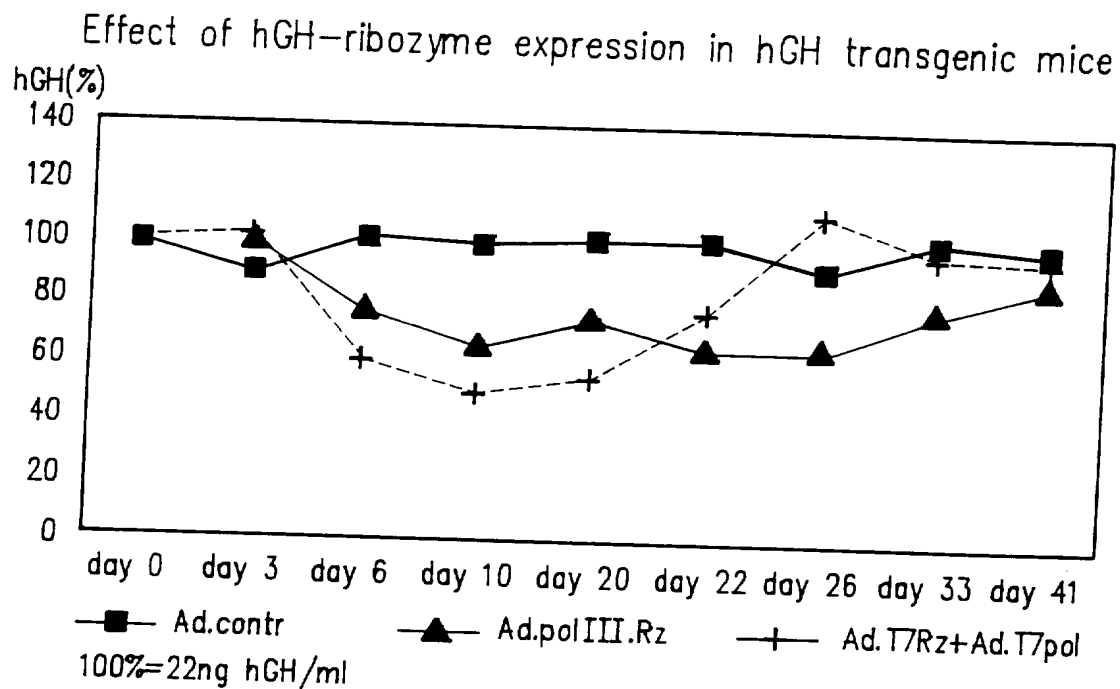
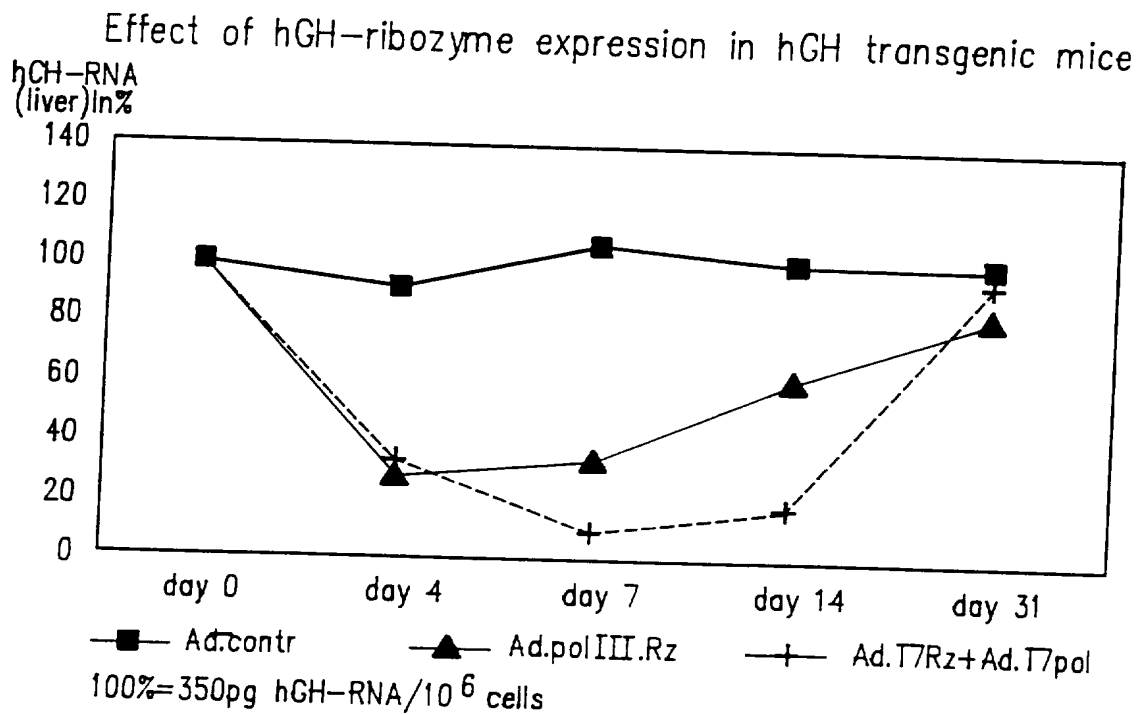
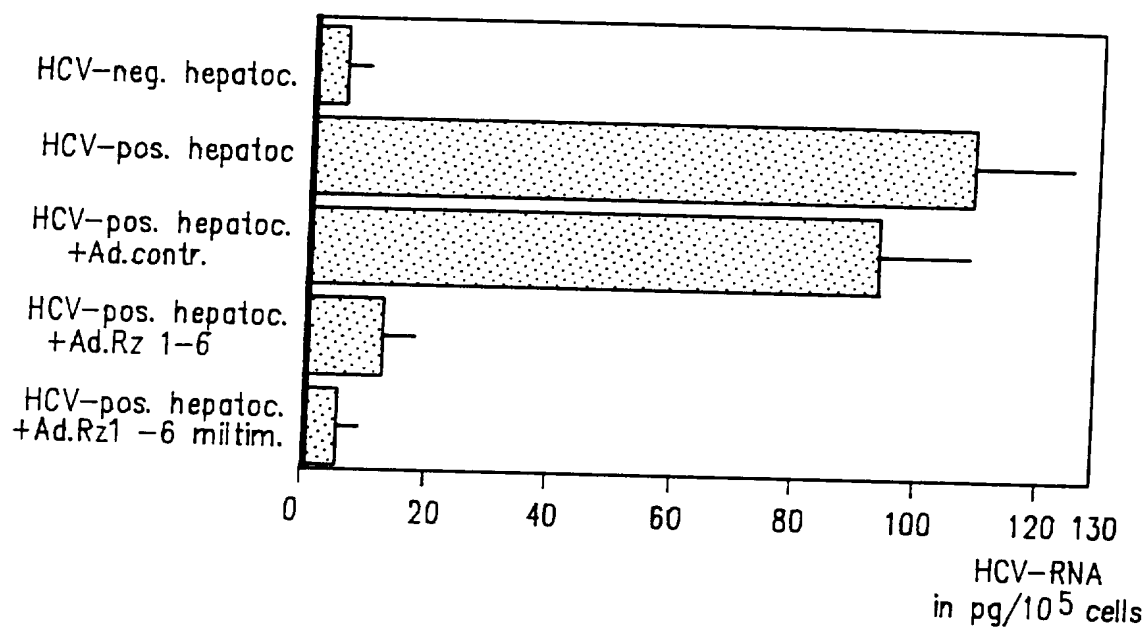


FIG. 11B



12/12

FIG. 12





# INTERNATIONAL SEARCH REPORT

international application No.  
PCT/US95/16347

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; C12N 15/00  
US CL : 514/44; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS MEDLINE BIOSIS EMBASE WPIDS CAPLUS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	Science, Volume 269, issued 25 August 1995, MARSHALL, "Gene Therapy's Growing Pains", pages 1050-1055, see entire document.	1-33
Y	Journal of Cellular Biochemistry, Supplement 0, 17 Part E, issued 1993, GEORGE ET AL, "Ribozyme Mediated Cleavage of Hepatitis B Virus Surface Antigen mRNA", page 212, abstract S407, see entire abstract.	15-33
Y	Journal of Cellular Biochemistry, Supplement 0, 17 Part E, issued 1993, SHIH ET AL, "A Novel Clamp-Like Structure Enables Retargeting of a Ribozyme Derived From the Antigenome of Hepatitis Delta Virus and Trans-Cleavage of Heterologous RNA", page 214, abstract S416, see entire abstract.	15-33

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 MARCH 1996

Date of mailing of the international search report

20 MAR 1996

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

ANDREW MILNE

Telephone No. (703) 308-0196

**INTERNATIONAL SEARCH REPORT**International application No.  
PCT/US95/16347**C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT**

<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to claim No.</b>
Y	Hepatology, Volume 18, Number 5, issued 1993, LEDLEY ET AL, "Hepatic Gene Therapy: Present and Future", pages 1263-1273, see entire document.	1-33
Y, P	Human Gene Therapy, Volume 6, issued August 1995, LIEBER ET AL, " A Modified Urokinase Plasminogen Activator Induces Liver Regeneration Without Bleeding", pages 1029-1037, see entire document.	1-33
Y	Science, Volume 263, issued 25 February 1994, RHIM ET AL, "Replacement of Diseased Mouse Liver By Hepatic Cell Transplantation", pages 1149-1152, see entire document.	1-33